ANALYSIS OF THE MULTISUBUNIT EXOCYST COMPLEX IN *SCHIZOSACCHAROMYCES POMBE*

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A THESIS SUBMITTED
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
TEMASEK LIFE SCIENCES LABORATORY
NATIONAL UNIVERSITY OF SINGAPORE
2003

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisor Dr. Mohan Balasubramanian for excellent mentorship and unflagging support through the years. It has been such a fortunate experience for me to have your guidance and encouragement.

I would like to thank my thesis committee members Drs Suresh Jesuthasan, Jianhua Liu, Uttam Surana and Alan Munn for the time and effort they have put in this project and helpful suggestions and comments on my thesis projects.

I thank all past and present members of Balasubramanian and Munn laboratories, especially Dr. Jianhua Liu's for his guidance when I was new in the lab, Mr. Tang Xie for the collaboration on the project and Drs Naweed Naqvi, Suniti Naqvi, Vicky Boulton and Kelvin Wong for the helpful discussions and useful suggestions. I also thank Drs Suniti Naqvi, Ventris D'Souza, and Jim Karagiannis for critical reading of this thesis.

I thankfully acknowledge National Science and Technology Board / Agency for Science, Technology & Research, Singapore (until July 2002) and subsequently internal funds from the Temasek Life Sciences Laboratory, Singapore.

Finally, I would like to thank my husband Fengwei, without whose loving support and encouragement this thesis would not be possible.

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ABBREVIATIONS

aa	Amino acids
bp	Base pair
BSA	Bovine serum albumin
cDNA	complementary DNA
DAPI	4’6,-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
dNTPs	deoxy Nucleotide triphosphates
DTT	Dithiothreitol
EDTA	Ethylenediamine tetra acetic acid
GFP	Green fluorescence protein
IPTG	isopropyl—D-thiogalactopyranoside
kb	Kilobase pairs
kDa	kilo Daltons
Leu	Leucine
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PMSF	Phenylmethylsulfonylfluoride
rpm	revolutions per minute
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TCA	Trichloroacetic acid
TE	Tris/EDTA
TEMED	N,N,N’ N’ –tertamethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
ts	Temperature sensitive
Ura	Uracil

SUMMARY

Cytokinesis is the final stage of the cell division cycle in which one cell is physically divided to form two daughter cells. In recent years, the fission yeast *Schizosaccharomyces pombe* has emerged as an attractive model organism for the study of cytokinesis. It divides using an actomyosin ring whose constriction may generate the force necessary to pinch off two daughter cells. The constriction of the actomyosin ring is coordinated with the centripetal deposition of a multi-layered division septum, composed of one layer of primary septum adjacent with two layers of secondary septa. Finally, degradation of the inner layer of the division septum, primary septum, results in the liberation of daughter cells. While numerous studies have focused on actomyosin ring and division septum assembly, little information is available on the mechanism of cell separation.

In chapter III of this study, the essential role of a multi-protein complex, exocyst, in cell separation is described. These exocyst proteins localize to regions of active exocytosis: at the growing ends of interphase cells and in the medial region of cells undergoing cytokinesis in an F-actin-dependent and secretion-independent manner. Using biochemical means, I show that Sec6p, Sec8p, Sec10p, and Exo70p interact physically with each other, indicating that these proteins form a complex *in vivo* in *S. pombe*. *sec8*-1, a temperature-sensitive mutant deficient in a component of the exocyst complex, is defective in cell separation, but not in other aspects of cytokinesis at restrictive temperature. Analysis of a number of mutations in various exocyst components has established that these components are all essential for cell viability. Interestingly, all exocyst mutants analyzed appear to be able to elongate and assemble division septa, but are defective for cell separation. I therefore propose that the fission

yeast exocyst may be involved in targeting of enzymes responsible for septum cleavage. It is possible that cell elongation and division septum assembly can continue with minimal levels of exocyst function. Interestingly, *sec8*-1 mutants accumulate ~100 nm vesicles in the cytoplasm, indicative of a defect in post-Golgi vesicle trafficking to the plasma membrane. In *sec8* shut-off cells, a similar accumulation of putative vesicles was also observed, suggesting that Sec8p, and by in extrapolation, the fission yeast exocyst, is likely to be involved in exocytosis.

Chapter IV describes the analysis of the role of Rho3p, a member of the Rho family of small GTPase, in cell separation and exocytosis. The *rho3* gene, which encodes a Rho family GTPase, was isolated as a high-copy suppressor of *sec8*-1, the exocyst component mutation (Wang et al., 2002). Overexpression of Rho3p suppresses cell separation and exocytic defects of *sec8*-1, suggesting a functional correlation of Rho3p with Sec8p. Overproduction of Rho3p also suppresses the temperature-sensitive growth phenotype observed in cells lacking Exo70p, another conserved component of the *S. pombe* exocyst complex. Cells deleted for *rho3* arrest at higher growth temperatures with two or more nuclei and uncleaved division septa between pairs of nuclei, indicative of a defect in cell separation. *rho3Δ* cells, like *sec8*-1 cells, accumulate ~100 nm vesicle-like structures in the cytoplasm, suggesting that Rho3p may play a role in exocytosis, similar to the exocyst complex. Taken together, these results suggest the possibility that *S. pombe* Rho3p regulates cell separation and exocytosis by modulation of exocyst function.

Key words: *S. pombe*, cytokinesis, exocytosis, exocyst complex, Rho3p

Chapter I Introduction

1.1 A general introduction to cytokinesis

1.1.1 Cytokinesis

Cytokinesis is one of the late events of the cell cycle, which physically partitions segregated chromosomes, organelles and cytoplasm, by building membranous barriers between the post mitotic nuclei leading ultimately to the generation of two independent daughter cells (recently reviewed by Guertin et al., 2000). In a number of eukaryotes, including yeast, nematode and mouse, cytokinesis is achieved through the use of an actomyosin-based contractile ring (Hales et al., 1999). In the majority of organisms the actomyosin ring is formed following entry into mitosis and subsequently constricts following mitotic exit. This constriction may generate the forces required to invaginate membranes at the cleavage furrow (Schroeder, 1990). In coordination with actomyosin ring constriction new membranes and cell wall (in some organisms) between the post mitotic nuclei are assembled to allow daughter cells to become fully independent of each other (Bowerman and Severson, 1999; Smith, 1999). Finally, the daughter cells are liberated during abscission /cell separation (Glotzer, 1997; Johnson et al., 1982).

1.1.2 Model organisms used to study cytokinesis

Studies of cytokinesis in animals, yeasts, plants and bacteria have greatly contributed to the understanding of this event. Cytokinesis is achieved using different mechanisms in different organisms (Fig 1.1.2). In animal cells cytokinesis is dependent on the assembly of an actomyosin ring (reviewed by Hales et al., 1999) followed by the addition of new membranes at the site of division concurrently with ring constriction (reviewed by Bowerman and Severson, 1999). In plant cells cytokinesis appears to not

involve the function of an actomyosin ring, but is achieved through the assembly of new cell wall and membranes at the division site (reviewed by Staehelin and Hepler, 1996). Yeast and fungal cells accomplish cytokinesis using three structures: the actomyosin ring, the division septum (a structure made up of cell wall polymers) and new membranes (reviewed by Guertin et al., 2002). Whereas events such as DNA synthesis and mitosis have displayed remarkable conservation among diverse eukaryotes, cytokinesis presents a unique challenge. A combination of the actomyosin dependent/independent and cell wall requiring/non-requiring mechanisms participate in cytokinesis in different organisms. Therefore, it becomes important to study cytokinesis using various experimental systems to gain a relatively complete understanding of the mechanisms regulating this cellular event.

1.1.2.1 Animal cells

Animals including *Xenopus laevis*, *Drosophila melanogaster* and *Caenorhabditis elegans* have been used as model systems to study cytokinesis. Animal cells choose the site of division by using either overlapping astral microtubules (Rappaport model; Devore et al., 1989) or midzone microtubules (antiparallel microtubules appearing from anaphase to cytokinesis; Wheatley and Wang, 1996) of the mitotic spindle. At the site of division, an equatorial actomyosin ring composed of actin, myosin (Schroeder, 1990) and a number of other proteins, is assembled in late anaphase. Most components of the actomyosin ring including myosin, anillin, PSTPIP, and septins appear to be conserved between yeast and animals (reviewed by Hales et al., 1999). Membranous barriers between daughter cells are formed during furrow ingression. In sea urchin, *C. elegans*, *Xenopus* and *Drosophila*, new membrane addition at the cleavage furrow is required for cytokinesis (Collas et al., 1996; Jantsch-Plunger and

Glotzer, 1999; Lecuit and Wieschaus, 2000; Loncar and Singer, 1995; Roberts et al., 1992). Furrow ingression terminates when it reaches the spindle midzone and two daughter cells remain connected by a cytoplasmic bridge (McIntosh and Landis, 1971). Finally, the remnants of the contractile ring and spindle midzone are disassembled, the cytoplasmic bridge is broken and the plasma membrane is sealed in the process of completion/abscission (McIntosh and Landis, 1971).

1.1.2.2 Yeasts

The fission yeast, *S. pombe,* determines the position of the division site by the position of the interphase nucleus (Chang et al., 1996). In early mitosis, the actomyosin ring is assembled at the medial region of the cell and undergoes constriction at the end of mitosis (Guertin et al., 2002). Concurrent with actomyosin ring constriction is the deposition of a division septum that is composed of cell wall material at the division site (Gould and Simanis, 1997). The division septum is a three-layered structure and is composed of an inner primary septum and two flanking layers of secondary septa (Gould and Simanis, 1997). The primary septum is composed of both linear and branched 1,3-β-glucans, whereas secondary septa is composed of linear 1,3-α-glucan, 1, 6-β-glucans and branched 1,3-β-glucans (Duffus et al., 1984; Humbel et al., 2001). Finally, cell separation occurs by the degradation of the primary septum by 1,3-α- and 1,3-β- glucanases that liberate the two daughter cells (Johnson et al., 1982). This is an event unique to unicellular fungal cells surrounded by rigid cell walls. Both the actomyosin ring and the division septum are essential for cytokinesis in *S. pombe*. The ease with which fission yeast can be manipulated experimentally, taken together with the similarities in the process of cytokinesis in fission yeast and animal cells, makes it

an attractive model to study the dynamics and assembly of the actomyosin ring as well as formation of new membrane/cell wall during cytokinesis.

In the budding yeast, *Saccharomyces cerevisiae*, the division plane is under the control of mating type loci and is specified by the formation of a septin ring at the cell cortex. Mating type **a** and α haploid cells assemble axially placed buds, while **a**/α diploid cells assemble bipolar buds (Chant and Pringle, 1995). A new bud grows upwards from the position of the septin ring which becomes the future site of cell division. The actomyosin ring is assembled in two temporally distinct stages. The Myo1p ring forms early in the cell cycle at the G1/S transition, and the actin ring assembles in late mitosis (Bi et al., 1998; Lippincott and Li, 1998). Cells lacking Myo1p are viable but do display cytokinetic defects, suggesting the actomyosin ring is not essential for cytokinesis in budding yeast (Bi et al., 1998). Hof1p/Cyk2p, the Pombe Cdc15 homology (PCH) family member, plays a role in Myosin II-independent cytokinesis (Lippincott and Li, 1998; Vallen et al., 2000). The actomyosin ring undergoes contraction after anaphase, concomitantly with the formation of the division septum (Bi et al., 1998). The deposition of chitin-rich septum is dependent on two chitin syntases, Chs2p and Chs3p (Shaw et al., 1991; Silverman et al., 1988). Chs2p plays a major role in septum formation and Chs3p only becomes important when Chs2p is depleted in the cell (Shaw et al., 1991). Both Chs2p and Chs3p localize to the division site, but at different stages of the cell cycle. Chs3p localizes early in the cell cycle and maintains its localization till late mitosis while Chs2p localizes only in late mitosis (Chuang and Schekman, 1996). Cell separation occurs by degradation of the septum by chitinases and β-1,3-glucanases (Kuranda and Robbins, 1991; Baladrón et al., 2002).

1.1.2.3 Plants

An actomyosin ring structure has not been observed in higher plants including *Arabidopsis thaliana* and maize. Cytokinesis in plants is achieved through the construction of a cell plate (new cell wall) sandwiched between new plasma membranes at the division site (reviewed by Staehelin and Hepler, 1996). The plane of division is determined in G2 and early mitosis by a preprophase band (PPB), composed of cortical microtubules and actin filaments (Wick, 1991). The PPB is thought to guide the formation of the phragmoplast during cytokinesis (Wick, 1991). The phragmoplast is structure at the cell equator composed of oppositely oriented microtubules, where Golgi-derived vesicles are transported and fuse together to form a cell plate (Smith, 1999). Several plus-end-directed microtubule motor, kinesins have been found to localize to the phragmoplast and are required for its organization (Smith, 1999). However, the motors responsible for the movement of these vesicles to the cell plate remain to be identified. A MAPK (mitogen-activated protein kinase) cascade is involved in cytokinesis probably by regulating the phragmoplast expansion (Nishihama et al., 2001; Nishihama et al., 2002). The phragmoplastin, a protein related to the budding yeast dynamin, is required for cell plate formation (Gu and Verma, 1997). The interesting possibility is that as in *S. cerevisiae*, it might also play a role in the budding of clathrin-coated vesicles during cell plate formation. The fusion of these post-Golgi vesicles contributes cell wall polysaccharides, proteins and membranes to the cell plate (Smith, 1999) and is dependent on a syntaxin (see chapter 1.3.4).

1.1.3 The mechanisms of cytokinesis

1.1.3.1 The spatial control of cytokinesis

Finding the correct site for placement of the division apparatus is crucial for the proper execution of cytokinesis. Yeast , animals and plant cells have evolved very different mechanisms in this spatial control. In *S. pombe* cells, the cytokinetic apparatus is assembled overlying the position of the nucleus (Chang et al., 1996). The budding yeast cells decide the plane of division according to their previous division site (Chant and Pringle, 1995). Plant cells determine the division plane by a network of microtubules and cortical actin filaments (Wick, 1991). In animal cells, astral microtubules were initially found to carry the signals to the cortex, which in turn stimulate the assembly and contraction of actin-myosin II filaments along the equator in animal cells (Rappaport model; Devore et al., 1989). Recently, the role of interzonal microtubules formed during anaphase composed of antiparallel microtubules that are bundled with their plus ends interdigitating has been appreciated (Wheatley and Wang, 1996).

1.1.3.2 Composition and assembly of the actomyosin ring

The key components of the ring are actin and the motor protein myosin II (Schroeder, 1990). Type II myosins are important for the assembly of the actomyosin ring and are likely to be responsible for the force of contraction of the actin filaments on the cell cortex (Kitayama et al., 1997; Motegi et al., 1997; Satterwhite and Pollard, 1992). It has been proposed that in the actomyosin ring, bipolar myosin filaments connect to each other by actin filaments. The 'walking' of myosins along the actin filaments then results in the contraction of the actomyosin ring in a process similar to that seen in

muscle cells (Satterwhite and Pollard, 1992). In addition, a large number of other conserved proteins are associated with the actomyosin ring and are required for its assembly. Many of these proteins are known to bind actin and regulate its polymerization (Guertin et al., 2002). However, the timing and the order of addition of various components differ among yeasts and animals (Guertin et al., 2002).

1.1.3.3 New membrane/cell wall formation

During cytokinesis, the surface area of cells at the division site must expand to allow formation of new cell membranes that will serve as boundaries for newborn cells (reviewed by Hales et al., 1999). In higher plants, the fusion of Golgi-derived vesicles to the plasma membrane at the division site builds up the new membrane/cell wall during cytokinesis (Staehelin and Hepler, 1996). For example, the *Arabidopsis* KNOLLE, a cytokinesis-specific syntaxin (see chapter 1.3.4), is required for this vesicle fusion event (Lauber et al., 1997). Similarly, in animal cells, syntaxins have recently been found to be required for cytokinesis, suggesting a direct role for membrane fusion in cytokinesis in animal cells. Syntaxins from *Drosophila* (Burgess et al., 1997), sea urchin (Conner and Wessel, 1999) and *C. elegans* (Jantsch-Plunger and Glotzer, 1999) are found to be important for cytokinesis. In budding yeast, membrane trafficking is involved in the formation of the septum. Two cell wall synthesizing enzymes, chitin synthase II and III are transported at least in part through the secretory pathway (Chuang and Schekman, 1996; Holthuis et al., 1998). The fusion factors that are involved in septum formation and new membrane addition in fission yeast have not been identified.

1.1.3.4 Cell separation

During cell separation/abscission, the cellular structure connecting daughter cells, either the primary septum in yeast cells, or the cytoplasmic bridge in animal cells, is disassembled, leading to the liberation of two daughter cells. The molecular mechanisms of abscission are essentially unknown. *C. elegans* Zen-4p (a mitotic kinesin-like protein) and Cyk4p (RhoGAP) on the midzone microtubules are required for the completion of cleavage furrow ingression. It was suggested that this complex might help to promote the disassembly of the actomyosin ring to facilitate cell separation (Kitamura et al., 2001; Raich et al., 1998). In yeasts, cell separation involves the regional erosion of adjacent cell wall and the dissolution of the primary septum to liberate daughter cells. In *S. cerevisiae*, the enzymes responsible for this process include a chitinase encoded by the *CTS1* gene (Kuranda and Robbins, 1991) and an endo-glucanase encoded by the *ENG1*/*DSE4* (Baladrón et al., 2002). Ace2p, a transcription factor, is involved in cell separation by regulating the expression of Cts1p (Colman-Lerner A, 2001; Weiss et al., 2002) and Eng1p (Baladrón et al., 2002). Several forkhead transcription factors are also found to be implicated in cell separation indirectly by regulation of *ACE2* gene expression (Pic et al., 2000). A few proteins required for cell separation in *S. pombe* have been identified (refer to chapter 1.2.4).

1.2 Cytokinesis in *Schizosaccharomyces pombe*

1.2.1 The fission yeast cell cycle

1.2.1.1 The cell cycle and its regulation

The fission yeast mitotic cell cycle is similar to that of higher eukaryotes, making it a particularly suitable model for the studies of cell division (recently reviewed by Moser and Russel, 2000). The vegetative cell cycle of *S. pombe* (Figure 1.2.1.1) consists of G1 (Gap), S (DNA Synthesis), G2 (Gap) and M (mitosis) phases (Mitchison, 1970).

During interphase (G1, S and G2 phases), fission yeast cells grow by elongation along their long axis (Pringle et al., 1997). Upon entry into mitosis, two SPBs (Spindle pole body) separate, forming a short mitotic spindle in the cell and sister chromatids align at the center of the spindle (McCully and Robinow, 1971; Uzawa and Yanagida, 1992). In anaphase A chromosomes segregate and start migrating toward the opposite SPBs (Uzawa and Yanagida, 1992), whereas in anaphase B, the spindle further elongate to fully separate the chromosomes to the ends of the cell (Uzawa and Yanagida, 1992). As in most fungi, the nuclear envelope of dividing *S. pombe* cells remains intact throughout; thus it undergoes a 'closed mitosis' (McCully and Robinow, 1971).

The cell cycle of the fission yeast is regulated by cyclin-dependent kinases (CDK), consisting of a catalytic kinase subunit Cdc2p and regulatory subunits cyclins (reviewed by Moser and Russel, 2000). This is a universal regulatory mechanism conserved among most eukaryotes (Norbury and Nurse, 1990). Entry into mitosis in fission yeast is triggered by high levels of Cdc2p-Cdc13p (Cdc13p, a B-type cyclin) (Hagan et al., 1988; Hayles et al., 1986). CDK activity at the G2-M boundary is regulated by the antagonizing activities of a phosphatase Cdc25p that stimulates CDK activity (Russell and Nurse, 1986) and a kinase Wee1p that inhibits CDK activity (Nurse, 1975).

1.2.1.2 Cytoskeletal rearrangement through the cell cycle

The major events in the fission yeast cell cycle are associated with the rearrangement of cytoskeletal structures including the microtubule and actin cytoskeletons (Figure 1.2.1.2).

1.2.1.2.1 Microtubule cytoskeleton

In interphase *S. pombe* cells, microtubules run along the long axis with their plus ends positioned at the cell tips and an overlapping region of minus ends at the medial region of the cell that overlies the interphase nucleus (Alfa and Hyams, 1990; Hagan and Hyams, 1988). The bipolar spindle is assembled in prometaphase/metaphase and elongates afterwards untill it reaches the ends of the cell to separate chromosomes. The mitotic spindle starts disassembly in late anaphase. This is followed by the formation of a PPA (postanaphase array of microtubules), which originates from MTOCs (microtubule organizing centre) at the medial region of the cell. Afterwards cells display an interphase array of microtubules. Although microtubules and the mitotic spindle play a critical role in cytokinesis in animals, they are not essential for cytokinesis in *S. pombe* (Arai and Mabuchi, 2002).

1.2.1.2.2 Actin cytoskeleton

The actin cytoskeleton plays a pivotal role in *S. pombe* cytokinesis (Marks and Hyams, 1985). The distribution of F-actin in *S. pombe* is linked intimately with the sites of growth and division (Alfa and Hyams, 1990; Marks et al., 1986; Marks and Hyams, 1985). Actin in fission yeast is observed as either patches or filaments (Marks and Hyams, 1985). During interphase, F-actin is mainly observed as patches that locate at the growing end(s) (Marks and Hyams, 1985). By early mitosis most of the actin patches disappear. Subsequently actin cables, together with a number of accessory proteins, form an actomyosin ring at the medial region of the cell cortex overlying the nucleus (Jochova et al., 1991; Marks and Hyams, 1985). Actin patches are subsequently polarized to the medial ring, which is thought to facilitate the formation of the division septum (Marks and Hyams, 1985). Following cytokinesis, F-actin

patches are relocated to the old end of the cell, and then to both ends of the cell, from where growth resumes (Marks and Hyams, 1985).

1.2.2 Mechanisms of cytokinesis in *S. pombe*

1.2.2.1 Positioning of the actomyosin ring in *S. pombe*

Mutants that are defective in positioning the actomyosin ring place the actomyosin ring randomly, and often tilted, which results in cells dividing asymmetrically (Chang et al., 1996). Mid1p/Dmf1p (a pleckstrin homology domain-containing protein) seems to inherit the positional cue from the nucleus and marks the position of cell division (Bahler et al., 1998; Sohrmann et al., 1996). Mid1p is localized in the nucleus in interphase. Upon entry into mitosis, it is phosphorylated by Plo1p (Polo-like kinase) and translocates from the nucleus to the medial cortex as a ring/band structure (Bahler et al., 1998; Sohrmann et al., 1996). Mid1p may then recruit actomyosin ring components at the central region of the cell cortex (Bahler et al., 1998).

1.2.2.2 Assembly of the actomyosin ring in *S. pombe*

In *S. pombe*, isolation and analyses of mutants defective in actomyosin ring assembly have identified a number of proteins that are involved in this process (Balasubramanian et al., 1998; Chang et al., 1996). They are Cdc3p (a profilin; Balasubramanian et al., 1994), Cdc4p (an EF-hand protein; McCollum et al., 1995), Cdc8p (a tropomyosin; Balasubramanian et al., 1992), Cdc12p (a formin; Chang et al., 1997), Cdc15p (an SH3 domain-containing protein; Fankhauser et al., 1995), Myo2p (a type II myosin; Kitayama et al., 1997; May et al., 1997; Naqvi et al., 1999), Rng2p (an IQGAP; Eng et al., 1998) and Rng3p (an UCS domain-containing protein; Wong et al., 2000). Genes encoding for these proteins are essential for cell viability and

conditional mutations (collectively named 'rng' mutants) in any of these genes lead to failure in the ability to form proper actomyosin rings and division septa, leading to the accumulation of multiple nuclei under restrictive conditions (Balasubramanian et al., 1998; Chang et al., 1996).

1.2.2.2.1 The role of myosin, light chains and an assembly factor

Myosin II, the F-actin based motor protein, plays a crucial role in the assembly of the actomyosin ring (Kitayama et al., 1997; Naqvi et al., 1999). *S. pombe* has two type II myosins, Myo2p and Myp2p (Bezanilla et al., 1997). Myo2p forms a dimer in which each heavy chain is associated with an essential light chain (ELC) and a regulatory light chain (RLC) (Le Goff et al., 2000; McCollum et al., 1995; Naqvi et al., 1999; Naqvi et al., 2000). Myo2p localizes to the actomyosin ring in early mitosis (Kitayama et al., 1997; Naqvi et al., 1999). Interestingly Myo2p is also observed as dot-like structures in interphase and early mitosis (Naqvi et al., 1999). The assembly of Myo2p into a ring structure requires F-actin (Naqvi et al., 1999). However, the accumulation of Myo2p dots at the medial region and the maintenance of Myo2p ring are independent of F-actin (Motegi et al., 2000; Naqvi et al., 1999). The Myo2p head region contains the motor domain and actin-binding domain, while the tail region contains a long coiled-coil domain that is required for dimerization (Kitayama et al., 1997; Naqvi et al., 1999). Analysis on mutations in the ATP-binding domain of Myo2p indicates that Myo2p motor activity is important for actomyosin ring assembly (Naqvi et al., 1999). Myp2p / Myo3p, the second myosin II in *S. pombe*, localizes to the actomyosin ring only in late mitosis, and is only required for cytokinesis under specific nutrient conditions (Bezanilla et al., 1997; Motegi et al., 1997). Myp2 is a

monomer and has two predicted coiled-coil regions that fold back on themselves to form a rod-shaped antiparallel coiled coil structure (Bezanilla and Pollard, 2000).

S. pombe Myo2p has two light chain binding sequences or IQ domains, namely IQ1 and IQ2 (May et al., 1997). *cdc4* encodes an EF-hand protein related to myosin light chains and is essential for cytokinesis (McCollum et al., 1995). Cdc4p is an actomyosin ring component and is physically associated with Myosin via its IQ1 domain, indicating that Cdc4p is an essential light chain for Myo2p (Naqvi et al., 1999). An *S. pombe* myosin regulatory light chain Rlc1p has also been found to be required for cytokinesis but only at lower temperatures (Le Goff et al., 2000; Naqvi et al., 2000). Rlc1p is observed as dot-like structures at the medial region in early mitosis and is localized to the actomyosin ring later in mitosis, reminiscent of that of Myo2p (Naqvi et al., 2000). Rlc1p seems to function by relieving the auto-inhibition of Myo2p, through binding to the IQ2 domain of Myo2p (Naqvi et al., 2000).

Rng3p is a member of the UCS domain containing protein family (UNC-45p Cro1p, She4p) and is likely to be involved in assembly of Myo2p as a molecular chaperone (Wong et al., 2000). Rng3p is observed as a ring structure at the division site of the *myo2*-E1 mutant but not in wild-type cells (Wong et al., 2000). It is thought that failed assembly of Myo2p in this mutant may trap more Rng3p at the division site to a detectable level (Wong et al., 2000).

1.2.2.2.2 A progenitor of the actomyosin ring

Besides their localization to the actomyosin ring in mitosis, Myo2p and Rlc1p are both observed as 'spot'-like structures in interphase cells (Wong et al., 2002). The Myo2p

and Rlc1p 'spot' colocalize, suggesting that they are components of the same 'spot' structures. Time-lapse studies show that during mitosis Myo2p appears as a spot at the division site and later as a few spots before forming into filaments and finally tightening into a ring structure, suggesting that the spot may represents the progenitor of the actomyosin ring. Interestingly, the Myo2p ring constricts into two spots at late anaphase, which seem to move into each daughter cell, suggesting that Myo2p spot may be inherited from the mother cell. Unlike Myo2p ring which undergoes rapid turnover, Myo2p spot does not seem to turn over, suggesting that they are physiologically distinct structures and the Myo2p spot may need to be inherited rather than assembled. Disassembly of the spot in interphase in a *rng3* mutant prevents the formation of the actomyosin ring in the subsequent round of mitosis, indicating that the Myo2p spot is important for the assembly of the actomyosin ring.

1.2.2.3 A SIN pathway regulating septation

In *S. pombe* septation (the initiation of actomyosin ring constriction and septum formation) is controlled by a signaling pathway termed the SIN (Septation Initiation Network, Figure 1.2.2.3).

The SIN is composed of Sid1p (a novel kinase; Guertin et al., 2000), Cdc7p (a kinase homologous to *S. cerevisiae* Cdc15p; Fankhauser and Simanis, 1994; Sohrmann et al., 1998), Spg1p (a small GTPase similar to *S. cerevisiae* Tem1p; Schmidt et al., 1997), Cdc11p (homologous to *S. cerevisiae* Nud1p; Krapp et al., 2001; Tomlin et al., 2002), Sid4p (a novel kinase; Chang and Gould, 2000), Cdc14p (a novel protein; Fankhauser and Simanis, 1993), Sid2p (a kinase homologous to *S. cerevisiae* DBF; Sparks et al., 1999), Mob1p (homologous to *S. cerevisiae*Mob1p; Hou et al., 2000). The analogous

Figure 1.1.2 A comparison of mechanisms of cytokinesis among animals, yeasts and plants. Animal cell cytokinesis is dependent on the assembly of the actomyosin ring and addition of new plasma membranes at the site of division in conjunction with ring constriction. Plant cell cytokinesis is achieved through the assembly of new cell wall and membranes at the division site. Yeast and fungal cells accomplish cytokinesis using all of the three structures- the actomyosin ring, the division septum made up of cell wall polymers and new plasma membranes

Fig 1.1.2

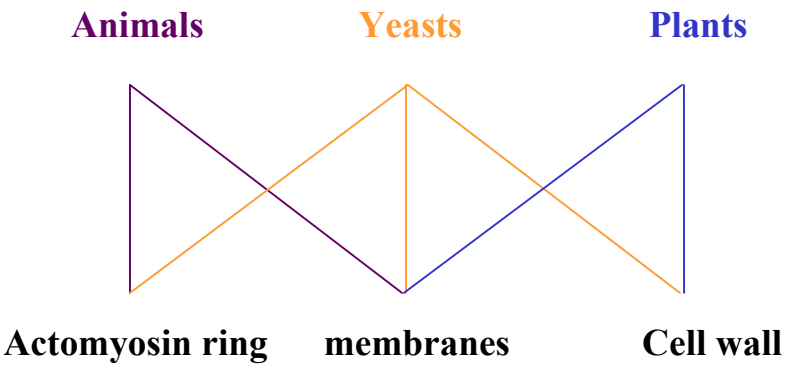

Animals
Yeasts
Plants
Actomyosin ring
membranes
Cell wall

Figure 1.2.1.1 The mitotic cell cycle of the *Schizosaccharomyces pombe*. The mitotic cell cycle of the fission yeast is consisted of four main stages, G1, S phase, G2 and M phase. During S phase, chromosome DNA is replicated and during mitosis, the replicated chromosomes are segregated. Cytokinesis occurs after M phase.

Figure 1.2.1.1

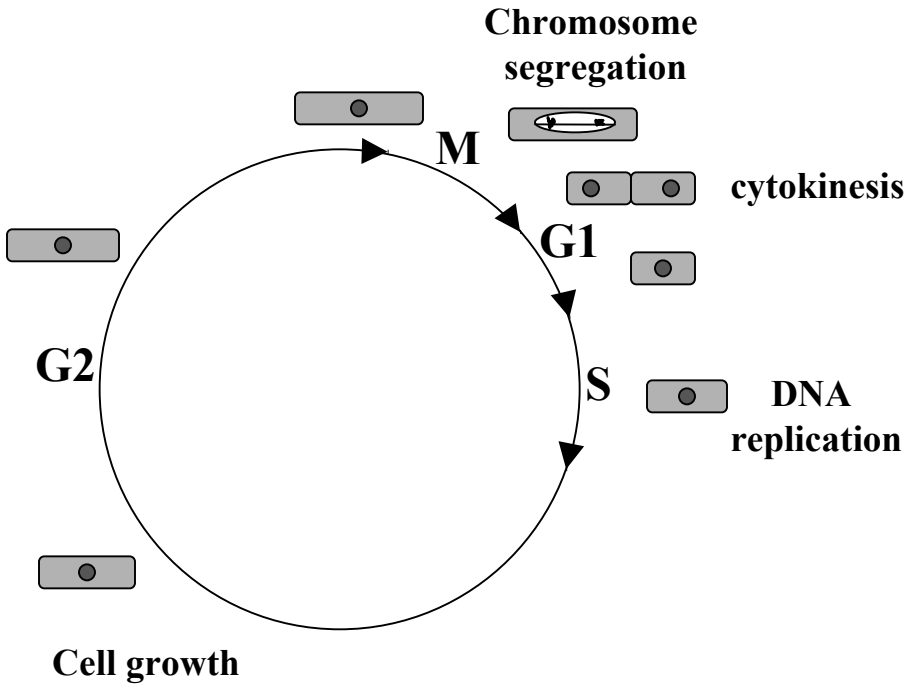

Chromosome segregation
M
cytokinesis
G1
S
DNA replication
G2
Cell growth

Figure 1.2.1.2 Cytoskeletal reorganization and cell wall construction during fission yeast cell cycle. In interphase, actin patches (red circles) concentrate at the growing ends of the cell. Upon entry into mitosis, actin patches disappear from cell ends and actin filaments are formed as a ring at the medial region of the cell. Actin patches are redistributed to the medial region to facilitate septum deposition. Concomitantly the actomyosin ring undergoes constriction, guiding the formation of new membrane and cell wall. After completion of mitosis, actin patches relocate to the old end of the cell, from where growth resumes. The interphase cell contains a cytoplasmic microtubule array. Concomitant with the disappearance of cytoplasmic microtubules at mitosis, the SPB (in yellow) separate to generates a short spindle. The spindle elongates as the cell undergoes anaphase and astral microtubules are also seen emanated from the SPBs. Following nuclear division, the mitotic spindle breaks down. Microtubule organizing centres at the equator of the parent cell nucleate new microtubules, forming the Post Anaphase Array (PAA). During cytokinesis, a division septum (in blue) is formed at medial region.

Fig 1.2.1.2

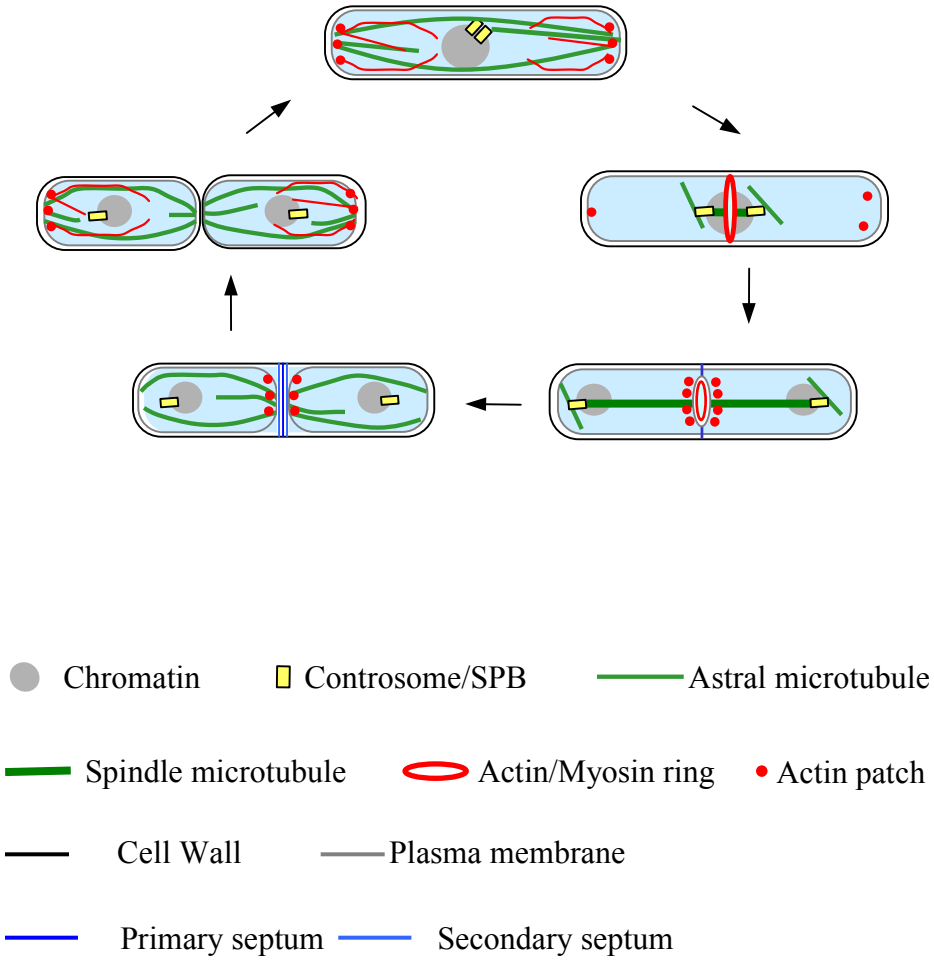
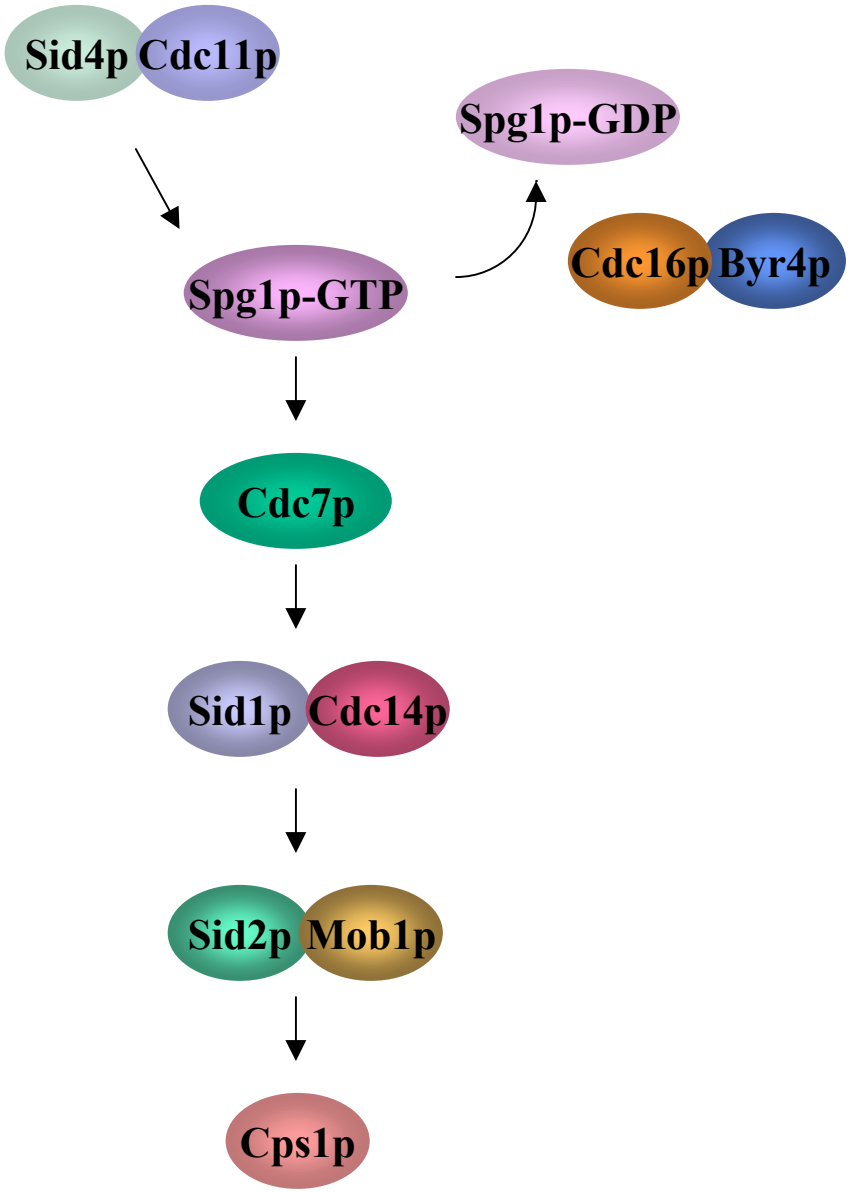

Chromatin
Controsome/SPB
Astral microtubule
Spindle microtubule
Actin/Myosin ring
Actin patch
Cell Wall
Plasma membrane
Primary septum
Secondary septum

Figure 1.2.2.3 The Septation Initiation Network (SIN) cascade. This signaling pathway regulates septation in *S. pombe*. The GTP-bound form of Spg1p lies at the top of the cascade. The GTPase activating protein (GAP) complex composed of Cdc16p and Byr4p regulates the conversion of the GTP-bound form of Spg1p to the GDP-bound form. This inactivates the signal from Spg1p and negatively regulates septation. Scaffold proteins Sid4p and Cdc11p are required for the localization of all other SIN proteins to SPB. The signal is transduced to Cdc7p, followed by Sid1p and Cdc14, and finally to Sid2p and Mob1p. Sid2p regulates the localization of Cps1p to the division site to initiate the septum formation.

Figure 1.2.2.3


Sid4p Cdc11p
Spg1p-GDP
Spg1p-GTP
Cdc16p Byr4p
Cdc7p
Sid1p Cdc14p
Sid2p Mob1p
Cps1p

pathway in *S. cerevisiae* is termed the MEN (mitotic exit network) which controls mitotic exit and has a role in cytokinesis (McCollum and Gould, 2001). All the *SIN* genes are essential and mutants deficient in them are capable of assembling an actomyosin ring in mitosis, but are unable to form a division septum and become multinucleated at restrictive temperatures (Balasubramanian et al., 1998; Nurse et al., 1976). The actomyosin ring in these mutants does not seem to contract and collapses after its formation, indicating that the assembly of the division septum is probably required for the stabilization and constriction of the actomyosin ring (Gould and Simanis, 1997).

A central component of this pathway is the Cdc7p kinase, which positively regulates septation in a dosage-dependent manner (Fankhauser and Simanis, 1994). Cdc7p associates with both SPBs during early mitosis (Sohrmann et al., 1998). During anaphase B, Cdc7p is localized asymmetrically to only one SPB and this persists in cells that are forming a division septum (Sohrmann et al., 1998). This asymmetric localization may be important to prevent the formation of more than one septum during cytokinesis (Sohrmann et al., 1998).

Cdc7p receives upstream signals from Spg1, a member of the ras family of small GTPases, to regulate septation (Schmidt et al., 1997). Spg1p is also a dosage-dependent inducer of septum formation and in its GTP-bound form may interact with Cdc7p (Schmidt et al., 1997). The GTPase activity of Spg1p is regulated by Cdc16p and Byr4p, a two-component GTPase activating protein (Fankhauser et al., 1993; Furge et al., 1998; Jwa and Song, 1998). Cdc16p and Byr4p are both negative regulators of septation (Fankhauser et al., 1993; Furge et al., 1998; Jwa and Song,

1998). Sid4p and Cdc11p, may act as a scaffold required for localization of other SIN proteins to SPB (Chang and Gould, 2000; Krapp et al., 2001; Tomlin et al., 2002).

Sid1p and Cdc14 function downstream of Cdc7p to signal Sid2p and Mob1p to trigger septation (Fankhauser and Simanis, 1993; Guertin et al., 2000; Salimova et al., 2000; Sparks et al., 1999). Both Sid1p and Cdc14p are asymmetrically localized to the same SPB as Cdc7p in late anaphase (Guertin et al., 2000). Sid2p localizes to the SPB throughout the cell cycle and is also localized as a band/ring at the division site at the end of anaphase, suggesting that Sid2p may transmit the signal from the SPB to the division site to trigger septation (Sparks et al., 1999). Mob1p has a very similar localization pattern as Sid2p, and these two proteins form a complex with each other to function in septation (Hou et al., 2000).

A likely target of SIN is Cps1p/Drc1p, a 1,3-β glucan synthase which is involved in the formation of the polysaccharide-rich division septum (Liu et al., 2002; Liu et al., 1999). SIN may control the septum formation by localizing Cps1p to the division site (Liu et al., 2002).

1.2.2.4 Cell separation in *S. pombe*

Approximately twenty mutants that are defective in cell separation have been isolated and analyses on them suggest that there are several independent ways to achieve cell separation in *S. pombe* (see below).

1.2.2.4.1 Transcription factors required for cell separation

Sixteen mutants, named from *sep1* to *sep16*, were isolated and found to be defective in cell separation in a genetic screen (Sipiczki et al., 1993). Several genes responsible for the mutations was cloned and found to be putative transcription factors (Ribar et al., 1997). *sep1* encodes a transcription factor homologous to the forkhead family of proteins that are found in several other organisms (Ribar et al., 1997). However, the *sep1* mutant has normal β-glucanase activities (Ribar et al., 1997), suggesting that the regulation of β-glucanase is independent of Sep1p function. *sep9* and *sep15* are similar to *S. cerevisiae* Spt8p and Med8p respectively, which are subunits of transcription complexes SAGA and Mediator (Sipiczki et al., 1999; Zilahi et al., 2000). Sep10p and Sep11p are also putative transcription factors. All of these 'Sep' proteins seem to play roles in multiple processes, e.g. sexual differentiation and are likely to affect cell separation indirectly (Grallert et al., 1999; Sipiczki et al., 1999). Most of the target genes regulated by these transcription factors remain to be identified. Recently, an ortholog of the ACE2 gene has also been shown to be involved in cell separation in fission yeast. *S. pombe ace2* mutants show a phenotype similar to *sep1* mutants. Ace2p has been shown to be required for the expression of Rng1p (Chapter 1.2.44) and thereby effecting cell separation (Martin-Cuadrado et al., 2003).

1.2.2.4.2 Mid2p stabilizes the septin ring during cell separation

Septins are a group of well-conserved GTPases identified in yeast and animals (Field and Kellogg, 1999; Kartmann and Roth, 2001). Several of them localize to the division site and are required for cell separation (Berlin et al., 2003; Tasto et al., 2003). Recently, Mid2p was identified based on its sequence similarity to *S. pombe* Mid1p and was found to be involved in cell separation (Berlin et al., 2003; Tasto et al., 2003). The septin ring in *mid2Δ* cells undergoes rapid exchange in FRAP (Fluorescence

recovery after photobleaching) analysis (Berlin et al., 2003) and in the presence of a nondegradable Mid2p, the septin ring is stabilized (Tasto et al., 2003), indicating that Mid2p stabilizes the septin ring during cytokinesis. The transcription of the *mid2* gene is dependent on Sep1p, suggesting that Sep1p may participate in cell separation by mediating the transcription of the *mid2* gene (Berlin et al., 2003; Tasto et al., 2003).

1.2.2.4.3 A few proteins are involved in cell separation through unknown mechanisms

A few other conserved proteins are also found to be involved in cell separation, but it is not known if their function is related to Sep1p/Mid2p/Septins. Ppb1p (Yoshida et al., 1994) is a calcineurin-like protein from *S. pombe*. *ppb1Δ* cells have mild defects in cell separation. *ppb1Δ sts1Δ* double mutant display severe cell separation phenotypes, suggesting Ppb1p may function related to Sts1p, which is homologous to chick Lamin B receptor (Shimanuki et al., 1992; Yoshida et al., 1994). Pob1p is homologous to budding yeast Boi proteins and is required for both polarized cell growth and cell separation in *S. pombe* (Toya et al., 1999).

1.2.2.4.4 A glucanase Eng1p participates in cell separation directly

While this work was carried out, an endo-β-1,3-glucanase Eng1p was identified and shown to be required for dissolution of the primary septum during cell separation in *S. pombe* (Martin-Cuadrado et al., 2003). Although the function of glucanases during cell separation has been anticipated for a long time, this finding was the first report in *S. pombe* that a glucanase indeed is directly involved in cell separation. *eng1Δ* cells show mild a defect in cell separation, suggesting that additional glucanases might be involved in cell separation in *S. pombe*. Eng1p has specific β-1,3-glucanase activities

in cell extracts, which peak during septation. Eng1p is localized to the septum during cytokinesis. Interestingly, a putative signal sequence is present in the amino terminus of Eng1p, which might be required for its entry into the secretory pathway.

1.3 Membrane dynamics during cytokinesis

1.3.1 An overview of secretory pathway

In eukaryotic cells, proteins whose destination is the cell surface are vectorially transported into endoplasmic reticulum (ER) and arrive at the plasma membrane, via the Golgi apparatus. The common features of transport from ER to Golgi and from Golgi to the cell surface are the assembly, targeting, docking and subsequent fusion of vesicle intermediates. Assembly of vesicle intermediates and their transport from ER to Golgi have been understood in great detail (recently reviewed by Gorelick et al., 2001). To maintain an ordered flow through the secretory pathway, pairs of membranes must specifically recognize one another and subsequently fuse.

1.3.2 A requirement for membrane dynamics during cytokinesis

To create barriers composed of new membranes between daughter cells, new membrane addition has to occur at the division site during cytokinesis (reviewed by Bowerman and Severson, 1999; Staehelin and Hepler, 1996). In addition, new cell wall material and/or enzymes responsible for synthesizing cell wall material must be delivered to the division site during cytokinesis in plants and fungi (Staehelin and Hepler, 1996). Newly synthesized membranes and cell wall need to be transported to the division site via vesicular intermediates followed by the fusion of the vesicles with the existing plasma membrane to increase surface area during cytokinesis (Bowerman and Severson, 1999; Staehelin and Hepler, 1996).

1.3.3 Exocytosis is utilized to achieve surface expansion during cytokinesis

Many eukaryotes appear to utilize exocytosis to achieve surface expansion during cytokinesis (reviewed by Bowerman and Severson, 1999). The accurate delivery of vesicles to the site of exocytosis requires targeted vesicle transport and subsequent docking and fusion of vesicles at specific sites of the plasma membrane (Waters and Pfeffer, 1999). In higher plants, new cell wall formed during cytokinesis results from the delivery of Golgi-derived vesicles from the microtubules of the phragmoplast. The fusion of these vesicles at the division site into the cell plate, which subsequently undergoes a series of maturation processes, becomes the new wall (Staehelin and Hepler, 1996). It has been proposed for animal cells that membrane vesicles are transported to the cleavage furrow by microtubule motors and fusion factors to ensure that vesicles are fused to the plasma membrane of the cleavage furrow (Straight and Field, 2000). The secretory pathway in budding yeast is required for the localization of cell wall-synthesizing enzymes to the division site during cytokinesis (Chuang and Schekman, 1996; Holthuis et al., 1998). However, the *S. pombe* secretory pathway is very poorly characterized and proteins that are involved in surface expansion during cytokinesis have not been identified.

The cytoskeleton plays a critical role in membrane trafficking during cytokinesis in most higher eukaryotes (Danilchik et al., 1998; Staehelin and Hepler, 1996). In higher plants, both microtubules and actin filaments are prominent cytoskeletal components of the phragmoplast (Staehelin and Hepler, 1996). Microtubules are essential for phragmoplast formation, but the precise role for actin filaments is unclear. In *Xenopus* embryos, membrane addition during cytokinesis is dependent on microtubules but

seems to be independent of actin (Danilchik et al., 1998). In contrast to animal cells, the vesicle transport in budding yeast involves only the actin cytoskeleton, but not microtubule cytoskeleton (reviewed by Finger and Novick, 1998).

1.4 The exocyst mediates tethering of vesicles at the plasma membrane

The late stages of secretion are the delivery and fusion of Golgi-derived vesicles with the plasma membrane (Jahn et al., 2003). Membrane vesicles are tethered close to the target membrane before fusion occurs (Waters and Pfeffer, 1999). Tethering factors are involved in the initial docking of the vesicles to their target membrane and are likely to control the spatial regulation of many membrane trafficking events (Waters and Pfeffer, 1999). The tethering factors (Whyte and Munro, 2002) identified so far are either fibrous proteins (i.e. *S. cerevisiae* Uso1p tethering vesicles from ER to Golgi) or multimeric protein complexes (i.e. TRAPP, transport protein particle, tethering vesicles from ER to Golgi). The exocyst from budding yeast is the best known tethering factor at a late stage of the secretory pathway (Hsu et al., 1999).

1.4.1 Exocyst is a multiprotein complex

In budding yeast the exocyst is a complex of eight proteins (Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p and Exo84p) that is involved in directing exocytic vesicles precisely to the specific sites of the plasma membrane (Hsu et al., 1999). All of the exocyst components are hydrophilic proteins and form a complex peripherally associated with the plasma membrane (Guo et al., 1999). The exocyst components localize to sites where active secretion occurs in the cell (Guo et al., 1999).

1.4.2 A targeting factor Sec3p

Sec3p in the exocyst complex may represent a spatial landmark for exocytosis and may be the component of the exocyst complex most proximal to the target membrane (Finger et al., 1998; Finger and Novick, 1997). The localization of Sec3p to the bud tip and division site is unaffected upon treatment with brefeldin A (BFA), a potent drug that blocks vesicle trafficking from ER to the Golgi apparatus. In addition, Sec3p localization is also independent of other components of the exocyst. Based on these observations, it was suggested that Sec3p localization is independent of ongoing secretion (Finger et al., 1998). Sec3p localization is also independent of actin-mediated trafficking (Finger et al., 1998). However, its localization is abolished in a mutant of the cyclin-dependent kinase, Cdc28p, in *S. cerevisiae* (Finger et al., 1998). It would be interesting to understand if Sec3p localization/activity is regulated through the mitotic CDK, Cdc28p (Finger et al., 1998).

1.4.3 A chain of interactions

To tether post-Golgi vesicles to the plasma membrane, the exocyst must not only interact with the target membrane, but must also be able to interact directly or indirectly with vesicles (Guo et al., 1999). A trail of protein-protein interactions is present to connect the secretory vesicles to Sec3p, the targeting factor of the exocyst localized to the plasma membrane (Guo et al., 1999). Direct interactions that have been identified include Sec3p-Sec5p, Sec5p-Sec6p, Sec5p-Exo70p, Sec10p-Sec15p, Sec5p-Sec10p and Sec6p-Sec8p (Guo et al., 1999). Except these interactions, all other pair-wise combinations tested were found to be negative (Guo et al., 1999). Each of the eight subunits has been found to exist as single copy in the exocyst complex, consistent with the hypothesis of ‘a chain of interaction’ in the exocyst complex (TerBush et al., 1996).

1.4.4 A subcomplex of Sec15p-Sec10p

Sec10p and Sec15p are not only present in the exocyst complex, but also form a subcomplex (Guo et al., 1999). This subcomplex seems to associate with the Golgi-derived secretory vesicles through the association of Sec15p with vesicles (Guo et al., 1999). The rab GTPase Sec4p acts as a molecular switch to regulate exocytosis and the majority of Sec4p resides on post-Golgi vesicles (Guo et al., 1999). Sec15p may associate with the secretory vesicles by the interaction with the GTP-bound form of Sec4p and through the chain of interactions to tether the secretory vesicles to the plasma membrane (Bowser and Novick, 1991; Guo et al., 1999).

1.4.5 The Sec6/8 complex in mammalian cells

Similar complexes, based on sequence homology, have been identified in different tissues of mammalian cells and are termed the Sec6/8 complex (Grindstaff et al., 1998; Hsu et al., 1996). In the epithelial Madin-Darby canine kidney (MDCK) cells, the Sec6/8 complex is required for the targeting of Golgi-derived vesicles to the plasma membrane (Grindstaff et al., 1998). Antibodies against rat Sec8p inhibit the delivery of low-density lipoprotein (LDL) receptors to the basolateral membrane, but not of p75 receptors to the apical membrane, in the polarized MDCK cells, suggesting that this complex plays a role in targeting vesicles to specific domains on the plasma membrane (Grindstaff et al., 1998). In cultured developing hippocampal neurons, Sec6/8 complex is present in the axons, dendritic and axonal growth cones (Hazuka et al., 1999; Murthy et al., 2003). This complex plays a role in recruiting and/or retaining synaptic-vesicles at active zones during synaptogenesis (Hazuka et al., 1999).

1.4.6 Rab GTPases

The exocyst proteins being soluble or peripheral membrane proteins need to interact with integral membrane proteins on the donor and target membranes to determine the targeting specificity (Waters and Pfeffer, 1999). Recent studies show that the exocyst interacts with various members of the Ras superfamily of small GTPase including Rab Rho and Ral protein in different organisms (Refer to Chapter 4.1). These interactions may contribute to the spatial and temporal regulation of vesicle tethering.

1.4.7 Fusion factors

The best-characterized proteins involved in membrane fusion events are the SNARE (soluble NSF attachment protein receptor) proteins, which play an essential role in mediating membrane fusion (reviewed by Linial, 1997). SNARE proteins (Linial, 1997) are located on vesicular compartment (v-SNARE, i.e. synaptobrevin/VAMP) or on target compartment (t-SNARE, i.e. syntaxins and SNAP-25 families). Interestingly, several cytokinesis-specific Syntaxins (t-SNARE) have been identified in animals and plants, suggesting an important role for syntaxins in cytokinetic vesicle fusion (Burgess et al., 1997; Conner and Wessel, 1999; Lauber et al., 1997; Lecuit and Wieschaus, 2000).

1.5 Rho GTPases that are involved in cytokinesis

Several Rho GTPases from yeast and animal cells have been implicated in cytokinesis. In *S. pombe*, a small GTPase Spg1p is a component of the SIN pathway that triggers septum formation (Chapter 1.2.3). Components of this signaling pathway have homologues in *S. cerevisiae* that might also function in cytokinesis (McCollum and Gould, 2001). The *S. cerevisiae* Spg1p homologue Tem1p binds to the contractile ring

component Iqg1p, making it a good candidate for receiving a temporal signal for cytokinesis and initiating actomyosin ring contraction (Shannon et al., 1999). In animal cells, the initiation of cytokinesis is thought to be triggered by activation of Rho family GTPases including Cdc42 and other Rho proteins (reviewed in Hales et al., 1999). A putative *Drosophila* Rho1 guanine nucleotide exchange factor is also required for the initiation of cytokinesis (Prokopenko et al., 1999). The Rho effectors Rho-kinase and citron kinase localize to the cleavage furrow in mammalian tissue culture cells (Madaule et al., 1998; Kosako et al., 1999). Rho GTPase may regulate downstream targets including IQGAPs, FH proteins (Formin homology) and RMLCs (myosin regulatory light chain) to effect cytokinesis (reviewed in Hales et al., 1999). Rho proteins also regulate myosin-light-chain kinases and myosin phosphatase which determine the phosphorylation state of RMLC and regulate contractility of the actomyosin ring during cytokinesis (reviewed in Hales et al., 1999).

1.6 Aim and objectives of this thesis

Isolation and characterization of mutants that are defective in cytokinesis have significantly contributed to the understanding of the mechanism of cytokinesis. However, despite the likely requirement for exocytosis during cytokinesis in *S. pombe*, mutants deficient in exocytic factors have not been identified through genetic screens for cytokinesis mutants. The molecular nature of this requirement remains elusive in *S. pombe*.

Aim and objectives of this study:

The broad aim of this study was to investigate the role of exocytic factors during cytokinesis in *S. pombe* using genetic and molecular means. The exocyst, a multi-

protein complex, is the best studied tethering factor that mediates the trafficking of cargo proteins from the Golgi apparatus to the plasma membrane. The objective of this thesis was to examine whether the fission yeast exocyst complex has any role in cytokinesis. A reverse genetic approach was taken to study the function of the exocyst in *S. pombe*, since the sequencing of *S. pombe* database has been completed and information about exocyst-like sequences in *S. pombe* was available in the database.

The exocyst in a number of organisms are regulated by small GTPases including rho, ral and ras. Rho3p, a member of small GTPase protein, was found to genetically interact with the exocyst in *S. pombe*. To better understand the mechanism of exocytosis and regulation of the exocyst in *S. pombe*, the function of Rho3p was analyzed by genetic and cell biological approaches.

1.7 Significance of this study

Although mechanism of cytokinesis has been well studied, the role of membrane trafficking in cytokinesis remains poorly understood. This study was the first attempt undertaken in this field to investigate the role of exocytosis-specifying factors during cytokinesis in the fission yeast and it was anticipated that it will shed light on relatively novel aspects of cytokinesis.

Chapter II Materials and Methods

2.1 Strains, reagents and genetic methods

2.1.1 *Schizosaccharomyces pombe* strains

S. pombe strains used in this study and their genotype are listed in Table I.

Table I *Schizosaccharomyces pombe* strains used in this study.

Name	Genotype	Source
MBY797	Sec6p-GFP-*ura4*$^+$ *leu1*-32 *h*$^-$	This study
MBY798	Sec10p-GFP-*ura4*$^+$ *leu1*-32 *h*$^-$	This study
MBY799	*cdc25*-22 Sec6p-GFP-*ura4*$^+$ *h*$^-$	This study
MBY800	*cdc25*-22 Sec10p-GFP-*ura4*$^+$ *h*$^-$	This study
MBY802	Sec6p-GFP-*ura4*$^+$ *leu1*-32 *h*$^+$	This study
MBY803	*sec6*::*ura4*$^+$/*sec6*$^+$ *leu1*-32/*leu1*-32 *h*$^-$/*h*$^+$	This study
MBY804	*sec10*::*ura4*$^+$/*sec10 leu1*-32/*leu1*-32 *h*$^-$/ *h*$^+$	This study
MBY810	*cdc15*-140 Sec10p-GFP-*ura4*$^+$	This study
MBY811	*cdc8*-110 Sec6p-GFP-*ura4*$^+$	This study
MBY812	*cdc12*-112 Sec6p-GFP-*ura4*$^+$	This study
MBY813	*cdc8*-110 Sec10p-GFP-*ura4*$^+$	This study
MBY814	*cdc12*-112 Sec10p-GFP-*ura4*$^+$	This study
MBY816	Hht-GFP-*ura4*$^+$ *leu1*-32 *h*$^-$	This study
MBY830	*cdc7*-24 Sec6p-GFP-*ura4*$^+$	This study
MBY832	*cdc7*-24 Sec10p-GFP-*ura4*$^+$	This study
MBY836	Sec10p-13myc-*ura4*$^+$ *leu1*-32 *h*$^-$	This study
MBY840	Sec6p-13myc-*ura4*$^+$ *leu1*-32 *h*$^-$	This study

MBY820	*sid2*-250 Exo70p-13myc-kan^{R} *ura4*-D18 *leu1*-32 *ade6*-21X h^{+}	This study
MBY843	Exo70p-13myc-Kan^{R} Sec10p-GFP-*ura4*$^{+}$	This study
MBY850	Sec6p-myc-*ura4* Sec10p-GFP-*ura4*$^{+}$	This study
MBY851	Sec10p-myc-*Ura4* Sec6p-GFP-*ura4*$^{+}$	This study
MBY852	Exo70p-myc-Kan^{R} Sec6p-GFP-*ura4*$^{+}$	This study
MBY856	Sec8p-GFP-*ura4*$^{+}$ *leu1*-32 h^{-}	This study
MBY861	*exo70*::*ura4*$^{+}$/*exo70 ura4*-/ *ura4*- *leu1*-32/*leu1*-32 h^{-} / h^{+}	This study
MBY864	Sec8p-GFP-*ura4*$^{+}$ Exo70p-myc-Kan^{R}	This study
MBY885	Sec8p-GFP-*ura4*$^{+}$ Sec6p-myc-*ura4*$^{+}$	This study
MBY886	Sec8p-GFP-*ura4*$^{+}$ Sec10p-myc-*ura4*$^{+}$	This study
MBY887	*sec8*-1 *ura4*-D18 *leu1*-32 h^{+}	This study
MBY936	*cdc25*-22, Gma12-GFP-*ura4*$^{+}$	This study
MBY888	*sec8*-1	This study
MBY965	*sec8*Δ-*ura4*$^{+}$/Sec8p-13myc-Kan^{R}-*leu1*$^{+}$, h^{-}/ h^{+}	This study
MBY972	*sec8*::pnmt81-*sec8*-*ura4*$^{+}$, *leu1*-32, h^{-}	This study
MBY809	*mid1*-18 Sec6p-GFP-*ura4*$^{+}$	This study
MBY831	*sid2*-250 Sec6p-GFP-*ura4*$^{+}$	This study
MBY833	*sid2*-250 Sec10p-GFP-*ura4*$^{+}$	This study
MBY841	Cdc15-HA-Kan^{R} Sec6p-GFP-*ura4*$^{+}$	This study
MBY842	Cdc15-HA-Kan^{R} Sec10p-13Myc-*ura4*$^{+}$	This study
MBY843	Exo70p-13Myc-Kan^{R} Sec10p-GFP-*ura4*$^{+}$	This study
MBY847	Cdc15-HA-Kan^{R} Sec10p-GFP-*ura4*$^{+}$	This study

MBY853	*cdc25*-22 Sec10p-Myc-*ura4*$^+$	This study
MBY863	Sec8p-GFP-*ura4*$^+$ *h*$^+$	This study
MBY865	Sec8p-GFP-*ura4*$^+$ Exo70p-Myc-Kan^R *sid2*-250	This study
MBY929	Sec8p-GFP-*ura4*$^+$ Myo51-Myc-*ura4*$^+$	This study
MBY930	Sec8p-GFP-*ura4*$^+$ Myo52-Myc-*ura4*$^+$	This study
MBY931	Spn1GFP-Kan^R Sec10p-Myc-*ura4*$^+$	This study
MBY932	*sec8*-1 Drc1-GFP-Kan^R	This study
MBY933	*sec8*-1 TA50-GFP-Kan^R	This study
MBY934	*sec8*Δ-*ura4*$^+$/Sec8$^+$ *leu1*-32 *h*$^-$/ *h*$^+$	This study
MBY964	*sec8*-1 *ura4*-D18 *leu1*-32 *h*$^-$	This study
MBY966	*sec8*Δ-*ura4*$^+$/Sec8p-GFP-Kan^R-*leu1*$^+$ *h*$^-$/ *h*$^+$	This study
MBY973	Sec8p-Myc-Kan^R-*leu1*$^+$ *ura4*$^-$ *h*$^+$	This study
MBY1274	Sec6p-13myc-*ura4*$^+$ *sec8*-1	This study
MBY1292	Spn1-GFP-Kan^R *sec8*-1	This study
MBY1366	*sec8*-1 Sec10p-GFP-*ura4*$^+$	This study
MBY1393	*rho3*Δ-*ura4*$^+$ Sec8p-GFP-*ura4*$^+$	This study
MBY1394	*rho3*Δ-*ura4*$^+$ Sec8p-Myc-Kan^R	This study
MBY1395	*spn1*Δ-Kan^R Sec8p-GFP-*ura4*$^+$	This study
MBY1403	*sec8*-1 Spn1-GFP	This study
MBY1443	*rho3*Δ Sec6p-GFP-*ura4*$^+$	This study
MBY1456	*sec8*-1 pREP42-GFP-*ypt2*	This study
MBY1303	*sec8*-1, pREP3-1-*leu1*$^+$	This study
MBY1304	*sec8*-1, pREP3-1-*pot1*-*leu1*$^+$	This study
MBY1305	*sec8*-1, pREP3-1-*rho3*-*leu1*$^+$	This study

MBY1325	*rho3*Δ::*ura4*$^+$, *h*$^+$	This study
MBY1370	*rho4*Δ::*ura4*$^+$, *h*-	This study
MBY1393	*rho3*Δ::*ura4*$^+$, Sec8p-GFP::*ura4*$^+$	This study
MBY1396	*sec8*Δ::*ura4*$^+$, pREP3-1-*rho3*-*leu1*$^+$	This study
MBY1443	*rho3*Δ::*ura4*$^+$, Sec6p-GFP::*ura4*$^+$	This study
MBY1452	pREP1-GFP-*rho3*-*leu1*$^+$, *ura4*-D18	This study
MBY1453	GFP-Rho3p::*ura4*$^+$, *leu1*-32,	This study
MBY 1459	pREP1-*rho3*-G25V-*leu1*$^+$, *ura4*-D18, *h*$^-$	This study
MBY 1460	pREP1-*rho3*-T30N-*leu1*$^+$, *ura4*-D18, *h*$^-$	This study

2.1.2. Media and growth conditions

Media used for vegetative growth (YES and MM) and genetic methods were as described in Moreno et al., 1991. Genetic crosses and sporulation were performed on YPD agar plates. Tetrad dissections were performed using a Singer MSM micromanipulator on YES or MM plates. Double mutants were isolated from Non-parental ditype (NPD) tetrad. Yeast transformations were carried out by LiAc method.

Latrunculin A (Molecular Probes Ins., L-12370) was used at 200 mM concentration. Thiamin was used at a final concentration of 5 μM to repress the transcription from nmt1 promoter (Maundrell, 1990). Brefeldin A (Molecular Probes, B-7450) was used at 100 μg/ml.

2.1.3 Plasmids

Plasmids used in this study are listed in Table II.

Table II

Name	Plasmid	Insert description
pCDL613	pREP3-1-*rho3*	
pCDL627	pJK148-*sec8*CT-GFP-Kan	*sec8*CT in KpnI-SmaI; GFP in SmaI-BamHI; Kan in BamHI-SacI. Frame: CCC GGG with SmaI. (from 625)
pCDL628	pJK148-*sec8*CT-13Myc-Kan	*sec8*CT in KpnI-SmaI; 13Myc-Kan in SmaI-SacI from pFA6a-13Myc-Kan. Frame: CCC GGG with SmaI.
pCDL630	pJK210-*sec10*CT-13Myc	Derived from pCDL 629 (SpeI deleted in 13myc). *sec10*CT in NotI-BamHI was linearized with SpeI.
pCDL631	pJK210-*sec6*CT-13Myc	*sec6*CT in NotI-BamHI.
pCDL651	pSK-*rho3* cDNA-#2	*rho3* cDNA in BamHI-EcoRI
pCDL653	pSK-*rho3*-GTP(25G->V) form of cDNA	in BamHI-EcoRI
pCDL654	pSK-*rho3*-GDP (30T->N) form of cDNA	in BamHI-EcoRI
pCDL671	pJK210-5'UTR-GFP-*rho3*NT	in SacI-NotI-NotI-xhoI for *rho3* 5' tagging of GFP.

2.1.4 Enzymes and drugs used

Restriction enzymes used were from New England Biolabs (Beverly, MA, USA). Both anti-mouse or anti-rabbit radish peroxidase (HRP)-conjugated secondary antibodies used for immunoblotting and immunoflorescence were from Sigma Chemical Company (St. Louis, USA). Rhodamine conjugated phalloidin, phloxin B (5mg/l) and lysing enzymes were from Sigma Chemical Company. Zymolyse was from US Biological (Swampscott, MA, USA). Glusulase was from NEN (Boston, MA, USA). Latrunculin was from Molecular Probes (Eugene, OR, USA).

2.2 Molecular methods and yeast methods

2.2.1 Standard recombinant DNA techniques were used in this study.

DNA digestions with restrictive enzymes were carried out at 37°C. Ligation was carried out at RT or 16°C for 1hr to overnight. DNA preparation and purification were performed using kits from Qiagen (Max-Volmer-Straße4, 40724 Hilden, Germany).

2.2.2 Nucleotide sequence determination

All nucleotide sequence determination (non-radioactive) was carried out using the Applied Biosystems prism 377 DNA sequencer (Forster, CA, USA).

2.2.3 Sequence comparison

Nucleotide and protein sequence homology searches were done using the BLAST program at the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

2.2.4 Transformation of *E.coli* by heat shock method

- 100-200µl *E.coli* competent cells were thawed on ice.

- 10 µl of DNA was mixed with the cells and incubated on ice for 10-30 min.
- Cells were heat shocked at 42°C for 45-60 sec and chilled on ice for 1 min.
- Cells were plated on LAB plate with Carbenicilin and incubated at 37°C overnight.

2.2.5 Southern blot

- 2-5 µg of genomic DNA (prepared as described above or extracted from Qiagen genomic DNA kit) was digested with appropriate enzyme for 1-2 hrs.
- The digested genomic DNA was run on an Agarose gel at 50 V for 2 hrs.
- The gel was slided into a plastic box, covered with depurination solution (250 mM HCl), and agitated for 10 min. Depurination solution was discarded and the gel was rinsed with distilled water.
- The gel was soaked with denaturation solution (0.5 N NaOH, 1.5 M NaCl) and agitated for 30 min. The denaturation solution was discarded and the gel was rinsed with distilled water.
- The gel was covered with neutralization solution and agitated for 15 min.
- DNA in the gel was transferred to a Hybond-ECL (Amersham) nylon membrane (pre-wet in distilled water and then soak in 10X SSC for 10 min) by setting up a capillary blot from bottom to top: (1) 3x Whatman 3MM paper soaked in 20xSSCP, (2) agarose gel turned upside down, (3) Saran wrap for sealing the sides, (4) Hybond-ECL membrane on the gel, (5) 3x Whatman paper, (6) paper towels and (7) weights. Transfer was done for overnight.
- Prehybridization was done on the membrane for 15min-1hr at 42°C .
- Probe labeling was performed by using a kit from Amersham. Briefly, 10 µl DNA (10 ng/µl) was denatured for 5 min in a boiling water bath, and immediately cooled

down on ice for 5 min. Equivalent volume of labeling reagent was mix with DNA thoroughly. The same volume of glutaraldehyde solution was then mixed thoroughly to the above mixure and incubated at 37°C for 10 min.

- Labeled DNA was added to prehybridization buffer and mixed gently to final concentration is 10 ng/ml. Hybridization was performed at 42°C overnight with gentle agitation.
- The membrane was washed with washing buffer (0.5 X SSC, pH 7.0, 0.4% SDS) twice, with 10 min per wash, at 55°C.
- The membrane was wrapped with Saran Wrap, and exposed to film for 1 min-1 hr.

2.2.6 LiAc transformation of *S. pombe*

- Cells were grown in 20 ml YE (for one transformation) overnight to 10^7 (OD595=0.5).
- Cells were washed with equal volume of water and spun down.
- Cells were the washed with 5 ml LiAc/TE buffer and resuspend in 100 µl of LiAc/TE [make 10 times dilution from 10X LiAC (1 M LiAc, pH 7.5) and 10X TE (0.1 M Tris-HCl, 0.01 M EDTA, pH 7.5) solutions].
- 2 µl of carrier DNA (10 mg/ml SSD) and 2-10 µl of plasmid DNA (10-20 µg) were added into 100 µl of cells and incubated at RT for 10 min.
- 240 µl of PEG/LiAC/TE solution (For 20 ml solution, dissolve 8 g of PEG 4000 in 2ml 10X LiAc and 2 ml 10 X TE and 9.75 ml water, and filter sterilize. This solution can be stored up to one month at 4°C) was added to the cells, gently mixed and incubated at 30°C for 10-30 min.

- 43 µl of DMSO was added into the cells and mixed by swirling. Cells were heated at 42°C for 5 min.
- Cells were then washed with 1 ml water and resusupend in 0.1 ml water to plate on selective plates.

2.2.7 Extraction of *S. pombe* genomic DNA

- 5 ml *S. pombe* cells were grown overnight at 24°C.
- Cells were spun down at 3000 rpm, washed once with in 0.5 ml TE.
- Cells were resuspended in 0.5 ml digestion buffer (1.2 M sorbitol, 0.1 M EDTA, 2 mg/ml Zymolyse, 1% BME) and incubated at 37°C for 30 min.
- Spheroplasts were spun out for 1 min at 3000 rpm, resuspend in 0.5 ml lysis buffer (100 mM NaCl, 50 mM EDTA, 50 mM Tris pH9.0. 50 µl 10% SDS and 10 µl 20 mg/ml proteinase K were added into lysis buffer before use), and incubated at 65°C for 30 min.
- Cells were transferred on ice for 10 min. 200 µl 5M Kac was added into cells, mixed by inversion, and keep on ice for 30 min.
- Cells were spun at 14, 000 rpm for 30 min at 4°C to remove proteins. Supernatant was recovered, and equal volume isopropanol was added to precipitate DNA. DNA was spun out at 14, 000 rpm for 10 min. The pellet was air dried for 10 min, resuspended in 40 µl TE containing RNAse and incubated at 37°C for 20 min.
- DNA could be keep at -20°C, and ready for PCR amplification.

2.2.8 Plasmid rescue from *S. pombe*

- Cells were harvested by scraping off a plate or from 5 ml saturated culture.

- Cell pellet was resuspend in 5X TE (50 mM Tirs pH 7.5, 5 mM EDTA.)
- Cells were spun down and the supernatant was removed as much as possible. 1 ml of glass beads were added (425-600 microns), and vortexed on high for 1 min. (use 15 ml snap cap tubes).
- 300 µl of lysis buffer (1% SDS, 100 mM NaCl, 10 mM Tris pH 8.0, 1 mM EDTA) was added to the tube.
- The liquid from the tube was extracted by vortexing the tube and removing liquid to an eppendorf tube. This was repeated once.
- 400 µl of phenol/ Chloroform was added to the eppendorf, the mixture was vortexed briefly and spun for 2 min. Aqueous (top) layer was transferred to a new eppendorf tube.
- 1 ml ethanol was added to the eppendorf tube, and the tube was spun for 5 min to precipitate DNA.
- The DNA pellet was washed with 70% ethanol, air dried for 5 min, and resuspended in 100 µl of TE.
- 2.5 µl of the solution was transformed into *E.coli* by electroporation and the remaining sample could be frozen at -20°C.

2.2.9 Spore germination

- Two *S. pombe* strains were crossed on a YPD plate at 28°C for 2 days.
- The plate was incubated at 18 °C for 1-2 days until most of the asci sporulated.
- Asci were harvested and resuspended in 5-10 ml 2% glusalase overnight at 25°C.
- Asci were checked under the microscope to ensure vegetative cells had been completely lysed after the digestion.

- Spores were washed 3 times with sterile water, could be stored at 4°C up to 2 months.
- Spores were inoculated at 30°C for 20-24 hrs in MM selective medium (liquid) at 10^7 to 10^8 spores per ml.

2.2.10 Synchronization by Nitrogen Starvation

MBY888 (*sec8*-1) was generated by crossing MBY887 (*sec8*-1, *ura4*-D18, *leu1*-32, h^+) to wild-type strain 972 (Leupold, 1970). MBY888 and wild-type cells were grown in minimal medium overnight at 24°C to early log phase (optical density$_{595}$ < 0.4). Cells were washed three times with minimal medium lacking nitrogen, resuspended in the same medium, and grown for 18 h at 24°C to arrest in the G1 phase. Cells were shifted to 36°C for 1 h to inactivate Sec8-1p and then transferred into YES (rich medium) to release cells from G1 and allow mitotic cell cycle progression at 36°C. Cell samples were taken just before the release (0) and every hour after the release (1–8).

2.3 Isolation of yeast genes, construction of knock out mutants and epitope tagging of genes

2.3.1 Identification of *sec6*$^+$, *sec8*$^+$, *sec10*$^+$, *sec15*$^+$ and *exo70*$^+$

A search through the Sanger Center Fission Yeast Genome Sequencing Project Database for proteins homologous to *S. cerevisiae* Sec6p, Sec8p, Sec10p, Sec15p and Exo70p identified *S. pombe* Sec6p, Sec8p, Sec10p, Sec15p and Exo70p. *sec6*$^+$, *sec10*$^+$, *sec15*$^+$ and *exo70*$^+$ were found to reside on cosmids SPCC1235.10c (Accession no. O74846), SPCC970.09 (Accession no. O74562), SPAC13F5.06c (Accession no.

O13705), SPCC1183.01 (Accession no. O75006) and SPBC582.02 (Accession no. Q10339), respectively.

2.3.2 Construction of deletion mutants for exocyst components

The entire coding sequences of *sec6*$^+$, *sec8*$^+$ and *sec10*$^+$ were deleted to create the null mutants by replacing the respective coding regions with the *ura4*$^+$ gene. The following primer pairs were used to amplify the constructs containing *ura4*$^+$ and the flanking 5' and 3' sequences of the respective gene by PCR. MOH595 (CCAGTCCGTAAATATATTAATCAATCTGTCAGTAAATAGAAACGTTTGT AAGCACTAGGTCTGCTTATAACTTTAAGAAAGCTACAAATCCCACTGGCTA TATGTA), containing 80 base pairs of *sec6*$^+$ 5'UTR region and 20 base pairs of 5' sequence of *ura4*$^+$, and MOH596 (GTAGATCATTAAAATTCAGCAACGACTACTTTGGATCGATATTGACGAAA CTTTTTGACATCATAATCAAAAGGAACATTACTATAGGTAAAGATAAACCG TAC), containing 80 base pairs of *sec6*$^+$ 3'UTR region and 20 base pairs of 3' sequence of *ura4*$^+$, were used to amplify the construct for to create *sec6Δ*. MOH597 (CACCTACAAACCAAAGGAACTTTGATCATTACTTTTCTATTCGAGAATTGT AGATTTAAAATTTCTTGTCTATTAAGACGCTACAAATCCCACTGGCTATAT GTA) and MOH598 (TATAATACACTATAAAAGATATTATGTTTATCTATAGACAAATTACTTCAT AATTAAGACATTAACAAAAATGAGCGATTGATATTGACGAAACTTTTTGAC ATC) were used for construction of the *sec10Δ*. The purified PCR products were introduced into a wild-type diploid *leu1*-32/*leu1*-32, *ura4*-D18/*ura4*-D18, *ade6*-210/*ade6*-216, h^+/h^-. *Ura*$^+$ transformants were checked for correct integration by PCR

assay and/or nucleotide sequence determination of the genomic DNA from transformants.

2.3.3 Epitope tagging and regulated expression of the exocyst gene products

Chromosomal copies of *sec6*$^+$, *sec8*$^+$, *sec10*$^+$ and *exo70*$^+$ were tagged by the carboxy-terminal addition of GFP and/or the Myc epitope. To tag Sec6p with GFP, a 0.8-kb KpnI/SmaI fragment of the *sec6*$^+$ C-terminal sequence was obtained by PCR using the primers MOH584 (GATGGTACCGAACTTTCACAGCAATTATCTG) and MOH585 (CGATCCCGGGTAAAATTGAACTTCCAGAAAGAG) and cloned into pJK210-GFP. The resulting plasmid pJK210-*sec6*CT-GFP, containing *sec6* in frame fused with GFP sequences, was linearized with NdeI and transformed into a wild-type strain of genotype *leu1*-32 *ura4*-D18 *ade6*-210. To tag Sec8p with GFP, primers MOH714 (CACCGGTACCAAGCTAATTTCGGTGGTGACTTT) and MOH715 (CTACCCCGGGATTTTTTCTCGCACCACCCACAG) were used to generate a 700-bp KpnI-SmaI fragment that was cloned into pJK210-GFP to yield pJK210-*sec8*CT-GFP. This plasmid was linearized with EcoRI and transformed into wild-type cells. Similarly, primers MOH586 (GATGGTACCTAGTGGACATTAGGGAATG) and MOH587 (CGATCCCGGGACTGCTCTTTGGGGGCAATAAAGCTTC) were used to generate a 0.9-kb KpnI-SmaI fragment of *sec10* that was cloned into pJK210-GFP to generate pJK210-*sec10*CT-GFP. This plasmid was linearized with SpeI and introduced into wild-type cells. In each case, transformants were selected on supplemented minimal medium lacking uracil, and putative integrants were subjected to PCR and/or Western blot analyses to confirm the desired integration. A similar strategy was used for Myc tagging. A 1.2-kb BamHI-BglII fragment containing the 13myc sequence and terminator from pFA6a-13myc (Bähler et al., 1998) was cloned

into the BamHI site of pJK210 (Keeney and Boeke, 1994) to generate plasmid pJK210-13myc. A 0.7-kb NotI/BamHI-digested fragment containing carboxyl-terminal sequence of *sec6*$^{+}$ was obtained using primers MOH638 (GCTAGCGGCCGCCCGAACTTTCACAGCAATTATCTG) and MOH639 (GCTAGGATCCGTAAAATTGAACTTCCAGAAAGAG) and was cloned into the NotI-BamHI sites of pJK210-*13myc*. Similarly, a 0.9-kb NotI/BamHI fragment of *sec10*$^{+}$ carboxy-terminal sequence was obtained using primers MOH623 (GCGAGCGGCCGCCCTAGTGGACATTAGGGAATGTAAG) and MOH624 (GCTAGGATCCGACTGCTCTTTGGGGGCAATAAAGC) and cloned into the NotI/BamHI sites of pJK210-*13myc*, in which the SpeI was deleted. These resulting plasmids were linearized with NdeI and SpeI, respectively, and transformed into wild-type cells. The desired integrations were confirmed by PCR assay and Western blot analysis. The tagging of *exo70* with 13myc was done according to the methods described by (Bähler et al., 1998) and was a kindly provided by Dr. Dannel McCollum.

2.3.4 Generation of mutations in *rho3*

A PCR based deletion strategy was utilized to construct the *rho3* null mutant. A pair of primers MOH730 (TGGTTCGCATCGCCTTAATTATTCTAAATTTGATTGTTACCTGAAATGTAG AAGCCAATCTACAGCGCACAAGGACATGTAATCCCACTGGCTATATGTA) and MOH731 (ACCCACACTAACGTCATATACAATAATAAACTTCGAACATTAGAAATAAG ACTTTTAGGCGCTTTCAAAAGAAAGTGCTTTAGGTAAAGATAAACCGTAC) were used to amplify a product containing the *ura4* gene as well as two flanking sequences of *rho3*. The PCR product was purified and transformed into wild-type

diploid cells to select on medium lacking uracil. Correct integration was confirmed by colony PCR analysis.

The mutant alleles, *rho3*$^{Val-25}$ and *rho3*$^{Asn-30}$ were constructed by an oligonucleotide-directed PCR. For construction of *rho3*$^{Val-25}$ mutation, primers with 18-mer overlapping sequences containing the mutation MOH738 (CTTGGTGATGTTGCTGCTGGTAAAACCAGTTTG), MOH739 (CCAGCAGCAACATCACCAAGAATTACGATTTTC) were designed to amplify *rho3* gene as two fragments with both carrying the mutation. These two fragments were mixed and used as templates for another round of PCR to generate a complete ORF of *rho3* with *rho3*$^{Val-25}$ mutation, which was subsequently cloned into pREP1. *rho3*$^{Asn-30}$ mutation was constructed by the similar strategy with primers MOH740 GCTGGTAAAAACAGTTTGTTAAATGTATTTACT) and MOH741 (TAACAAACTGTTTTTACCAGCAGCACCATCACC).

2.3.5 GFP tagging of Rho3p

rho3 cDNA was amplified using a *rho3* cDNA from pREP3x cDNA library as a template and amplified using primers MOH744 (CATGGATCCCATATGTCCTTCGCTAAGCACTG) and MOH745 (GGATCCCGGGTCAAGCAATGATACATCCGGTAC). The PCR product was cloned into pREP1-GFP as a BamHI-SmaI fragment to generate the plamid pREP1-GFP-*rho3*, which was subsequently transformed into a wild-type strain and colonies were grown on medium lacking leucine but contain thiamin.

2.4 Protein and immunological methods

2.4.1 Extraction of protein from *S. pombe* by a bead beater

- 50 ml *S. pombe* cells were grown up overnight until OD_{595}=0.5.
- Cells were spun down and washed once with 1 ml NP40 buffer (with proteinase inhibitors: 2 mM PMSF, 2 mM Benzamidine, 50 mM NaF, 100 μM Na_3VO_4, 4 μg/ml leupeptin).
- Cells were transferred into 1.5 ml screw capped eppendorf tube and spun down again. The supernatant was removed as much as possible.
- 1 ml acid wash glass beads (450-600 nm) were added into the tube. Cells were pulsed in a mini-bead beater for 3 times, 30 seconds duration and 2 min intervals.
- To prepare protein lysate, 300 μl of SDS lysis buffer (10 mM $NaPO_4$ pH 7.0, 0.5% SDS, 1 mM DTT, 1 mM EDTA,) was added to the tube and immediately boiled at 95°C for 2 min. Another 300 μl of NP40 buffer was added before extracting proteins from the tube. 200 μl of 4X sample buffer was mixed with protein lysates and boiled at 95°C for 2 min.
- To obtain native proteins, 300 μl of NP40 buffer instead of lysis buffer was added to extract soluble proteins. Cells extracts were spun at 14,000 rpm for 10 min, and ready for immunoprecipitation.

2.4.2 Preparation of protein lysate from *S. pombe* (NaOH lysis methods)

- 10-50 ml *S. pombe* cells were harvested at OD_{595}=0.5.
- Cells were washed once with cold 1X TE and transfer to eppendorf tube.
- Cell pellet was resuspended in 240 μl of 1.85 N NaOH, 1.06 M β-mercapoethanol (222μl of 2N NaOH, 18 μl of 13.97 M of β-mercapoethanol) and incubated on ice for 10 min.

- 15% volume of 100% TCA (36 µl for 240 µl) was added to the lysed cells to precipitate proteins and incubated on ice for 10 min.
- Proteins were spun down for 10 min at 14,000 rpm at 4°C. Protein pellets were washed with cold acetone and spin for 10 min. Repeat washing with acetone.
- The protein pellet was air dried for 10 min, and resuspend in 1X sample buffer.

2.4.3 Immunoprecipitation

- Protein extracts were prepared as described above.
- 2-5 µl of antibodies were mixed with 500 µl of protein extracts by inversion and the mixture was placed on ice for 1 hr with periodic mixing.
- 90-100 µl of Protein A Sepharose or 50 µl of Protein G Sepharose was added and incubated at 4°C on a nutator for 30min-1 hr.
- Sepharose beads were spun down at 5000 rpm for 1 min, and the supernatant was discarded.
- Sepharose beads were washed with 1 ml NP40 buffer with proteinase inhibitors and spun down. Repeat washing 5 times.
- 40 µl of 2 X Sample buffer was added to the Sepharose beads, boiled at 95°C for 3 min, and spun down in a microfuge briefly. The supernatant was loaded on a SDS-PAGE gel.

2.4.4 Protein electrophoresis, immunoblotting and detection

- After electrophoresis, proteins were transferred (Transblot-system, Bio-Rad Laboratories) to nitrocellulose membrane (Pierce, Chemical Co., Rockford, IL) at 85 V for 2 hr or at 35 V overnight.

- The membrane was blocked with 5-10% milk in PBS-Tween buffer (Tween20 0.1% in PBS) for 1 hr.
- Primary antibody was added at 1:1000 dilution or 1μg/ml in PBS-Tween, and incubated for 1-2 hrs.
- The membrane was washed with PBS-Tween for 3 times, 10 min each.
- HRP-conjugated secondary antibody was added at 1:10,000 dilution or 10 ng/ml for 1 hr.
- The membrane was washed with PBS-Tween 20 for 3-6 times, 10 min each.
- ECL detection agents were prepared (200 μl of 250 mM Luminol in DMSO**,** 88 μl of 90 mM P-coumaric Acid in DMSO**,** 4 ml 1 M Tris-HCl pH 8.5, 12 μl of 30% H_2O_2, 36 ml H_2O).
- The membrane was incubated in the detection agents for 1 min.
- Excess reagents were removed. The membrane was sandwiched by two pieces of clean plastic wrap.
- X-ray film was expose to the membrane and developed.

2.4.5 Stripping and reprobing the immunoblot

- To strip off the immunodetection system, the membrane was incubated in a standard stripping buffer (2% SDS, 62.5 mM Tris-HCl pH 6.8, 100 mM β-mercapoethanol) for 30 min-1 hr at 50-70°C.
- The membrane was washed with TBS-Tween, twice for 10 min per wash.
- The membrane was again blocked in blocking buffer as described above and continue Western blot protocol.

2.4.6 Measurement of Acid Phosphatase Secretion

Acid phosphatase secretion was assayed as follows [modified from (Craighead et al., 1993; Tanaka and Okayama, 2000)]. Because up to 40% of acid phosphatase is secreted into the medium in fission yeast, enzyme activity was assayed in the culture supernatant. Cells were grown to log phase in minimal medium (MM) at 24°C, spun down, washed twice with MM, and resuspended in fresh MM at 24 or 36°C. Samples were taken at 0 h (time of resuspension) and at hourly intervals thereafter. For each sample, approximately 1 ml of culture was centrifuged (the amount of cells were normalized to $0.5X10^7$ cells using medium), and 500 µl of the supernatant was added to 500 µl of substrate solution (2 mM p-nitrophenyl phosphate, 0.1 M sodium acetate, pH 4.0; prewarmed to 30°C) and incubated at 30°C for 5 min. Reactions were stopped by the addition of 500 µl of 1 M sodium hydroxide. The absorbance at 405 nm was measured, using the 0 h sample as a blank control.

2.5 Microscopy

2.5.1 DNA, F-actin and cell wall staining

- 1-2 ml of *S. pombe* cells were fixed in 3.7% formaldehyde at growing temperature for 1 min.
- Fixed cells were washed with 1 ml of PBS and can be stored at 4°C for up to 2 months in PBS.
- Cells were washed with 1 ml PBS with 1% TritonX-100.
- Cells were washed twice with 1 ml PBS. Cells were resuspended in 20-30 µl of 1µg/ml DAPI (4'6,-diamidino-2-phenylindole) mounting medium [50% glycerol, 1 mg/ml *p*-phenylenediamine (antifade), 1 µg/ml DAPI].

- 0.5-1 µl of 0.1 mg/ml Rhodamine-conjugated Phalloidin solution in PBS was added.
- For cell wall staining, 1-2 ml fixed *S. pombe* cells were spun down, washed with 1 ml PBS, and resuspended in 30-100 µl of 5 µg/ml Calcofluor solution in PBS.
- 2-3 µl cells were spotted on the slide, and viewed under a fluorescence microscope.

2.5.2 Immunofluorescence staining

- 50 ml *S. pombe* cells were cultured overnight until OD_{595}=0.2-0.5.
- For formaldehyde fixation, 5 ml 37% formaldehyde was added into 20 ml cells, mixed by inversion and incubated at the growth temperature for 20-30 min. For methanol fixation, 20 ml cells were spun down and resuspended in 8 ml cold (-20°C) methanol for 8 min.
- Cell pellet was washed with 20 ml PBS twice and kept on ice. Cells can be stored in PBS at 4°C for up to 1 month.
- Cells were spun down (equal to original 5-10 ml cells), washed once with 1.2 M sorbitol in PBS and resuspended in 450 µl 1.2 M sorbitol in PBS.
- 50 µl of protoplasting buffer (50 mg/ml Lysing enzyme, 30 mg/ml Zymolase, 1.2M sorbitol in PBS) was added to cells and incubated at room temperature for 5-10 min. Progress of cell wall digestion was checked by viewing cells under the microscope (4 µl cells +0.5 µl 10% SDS).
- The reaction was stopped by filling the tube with 15-50 ml PBS+1% Triton. Spheroplasts were spun down and transferred to an eppendorf.

- Spheroplasts were washed with PBS and then with PBAL buffer (1% BSA, 100 mM Lysine-Hydrochloride, 50 μg/ml Carbenicilin, 1 mM Sodium Azide, in PBS). Block in 1 ml PBAL at RT for 1 hour.
- Spheroplasts were resuspended in 200 μl PBAL with 1:100 dilution of primary antibodies. They were incubated at RT with shaking for 3 hrs, or overnight at 4°C.
- Spheroplasts were washed 3 times with 500 μl PBAL and resuspended in 200 μl PBAL with 1:100 dilution of secondary antibodies. They were incubated at RT with shaking for 1 hr.
- Spheroplasts were then washed 3 times with 500 μl PBAL and resuspended in 30 μl of PBAL.
- 5 μl of cells were spread on a coverslip evenly with the pipette tip and were dried for several min.
- 1 μl of 1X DAPI solution was dropped on the microscope slide. The coverslip with Spheroplasts was covered on the slide. Spheroplasts were viewed under the fluorescence microscope.

2.5.3 Electron Microscopy to visualize the membrane structures

- 5ml *S. pombe* cells were spun down and washed three times with sterile water;
- Cells were fixed in 10ml 2% (wt/vol) potassium permanganate for 40 min at room temperature;
- Cells were washed for three times with sterile water, and resuspended in 50% ethanol for 20 min. Cells were then resuspended in 70% ethanol overnight at 4°C;
- The samples were dehydrated by sequential washes in :

70% ethanol, twice for 15 min;

90% ethanol, twice for 15 min;

100% ethanol, three times for 20 min at room temperature;

- The pellet was resuspended in propylene oxide for 10 min;
- The pellet was incubated in 1:1 mixture of propylene oxide and Epon resin for 1 hour and neat Epon resin twice for 2 hours;
- The sample was embed in Epon resin at 65°C overnight;
- Sectioning;
- The samples were stained with uranyl acetate and lead citrate.

2.5.4 Confocal microscopy

1-2 µl of cells were spotted on the borosilicate glass slide (Matsunami Trading, Japan), and were covered with a cover slide (Matsunami Trading, Japan). Cells were viewed using a LEICA DMIRBE inverted microscope equipped with a LEICA N Plan 100X/1.25 oil objective and an Orca II C4742-98 CCD (charge-coupled device) camera (Hamamatsu). Images were taken using LSM 510 software, and processed with Metaview (Universal Imaging Corporative, PA) or NIH image 1.62 (Bethesda, Maryland). 3D view images were taken by stacking Z-stack images.

Chapter III Characterization of the exocyst complex in *S. pombe*

3.1 Introduction

Cytokinesis in *S. pombe* is achieved through the use of the actomyosin ring. In addition, *S. pombe* cells divide by targeted membrane addition and formation of a division septum. In *S. pombe*, a β-1,3-glucan synthase, Cps1p, is a transmembrane protein responsible for septum formation (Liu et al., 1999), suggesting that Cps1p might be targeted to the division site through exocytosis. However, the exocytic factors that are required for the targeting of Cps1p or new membrane addition during cytokinesis were unknown. In this study, the role of an exocytic factor, the *S. pombe* exocyst in cytokinesis was investigated.

The exocyst in budding yeast is a large complex important in targeting but probably not fusion of Golgi-derived secretory vesicles to the plasma membrane (TerBush et al., 1996). It is the best identified tethering factor in exocytosis. Unlike t-SNAREs [target SNARE (soluble NSF attachment protein receptor) proteins] that are evenly distributed along the plasma membrane, the exocyst is specifically localized at sites of active secretion. The exocyst is localized to the pre-bud site early in the cell cycle and later recruited to the bud tip, where polarized membrane addition takes place. During cytokinesis, this complex is located to the mother-daughter cell boundary, where membrane expansion is required for formation of septum (TerBush et al., 1996). This localization suggests a potential role for the exocyst in cytokinesis, although no direct investigations have been done on this issue. A similar complex named the Sec6/8 complex has been identified in animals. The Sec6/8 complex is expressed ubiquitously in multiple tissues and a mutation in *sec8* in mice causes early embryo lethality, suggesting that components of the exocyst may be conserved across several

species. Microinjection of monoclonal antibodies against rat Sec8p into MDCK cells inhibited vesicle transport to the basolateral plasma membrane domain of these cells, indicating that the Sec6/8 complex may function in targeting and docking of secretory vesicles to the appropriate sites of the plasma membrane. As the exocyst appears to be important in membrane transport on appropriate domains, but not on all plasma membrane, I investigated whether it may be involved in targeting vesicles to the site of cell division in *S. pombe*.

In this chapter, a *S. pombe* exocyst complex was identified and its role in cytokinesis was determined. By analysis of a temperature-sensitive mutant *sec8*-1, a number of null mutants of the exocyst and a *sec8* shut-off mutant, it was concluded that these mutants are defective only in disassembly of the division septum, but not in actomyosin ring assembly or septum formation. Intracellular localization of the exocyst, was characterized with detailed studies on their localization dependencies, leading us to conclude that the exocyst is targeted to the site of division in an F-actin dependent manner, but is likely independent of exocytosis. It was also shown that the fission yeast exocyst has a conserved function in exocytosis probably by targeting secretory vesicles to the plasma membrane.

Of the work reported in this thesis, the *sec8*-1 mutant and two complementing plasmids were isolated by Mr. Tang Xie and the *exo70* gene was myc-tagged by Susanne Trautmann in Dr. Dan McCollum's laboratory (Wang et al., 2002). I had independently identified the exocyst components by searching the *S. pombe* genome sequence information and have carried out the analysis of all mutants (including *sec8*-

1), the biochemical analyses of the exocyst, its intracellular localization and dependencies.

3.2 Results

3.2.1. Linkage analysis of *sec8* gene

mut2-1 was identified as a temperature-sensitive mutant defective in cytokinesis (Tang, X., and Balasubramanian, M.K., unpublished results) and a plasmid containing *sec8* gene was able to complement the growth defect of *mut2*-1 at 36°C (Wang et al., 2002). To confirm that *sec8* was the gene mutated in the *mut2*-1 strain , I tested whether *mut2*-1 was linked to the *sec8* locus. The *sec8* chromosome locus was tagged with GFP (green fluorescent protein) epitopes and marked with *ura4*$^+$ to generate a Sec8p-GFP strain, and this strain was crossed to *mut2*-1. Tetrad analysis of the diploid showed that in their progeny (20 tetrads were analyzed), all colonies that were temperature-sensitive (ts) were *ura4*$^-$, and all non-ts colonies were *ura4*$^+$ (Fig 3.2.1), suggesting that no recombination occurred between *mut2*-1 and *sec8* locus. Thus, *mut2*-1 is tightly linked to the *sec8* gene. *mut2*-1 was referred to as *sec8*-1 thereafter in this study.

3.2.2 Sec8p is related to proteins from other organisms

The amino acid sequence of Sec8p was used as a query to search for related proteins using the BLASTP program. *S. pombe* Sec8p is most closely related to the putative exocyst complex component Sec8p from the filamentous fungus *Neurospora crassa*. These two proteins share 22% identity and 40% similarity along their entire length (Fig 3.2.2). It is also related to the budding yeast *S. cerevisiae* Sec8p, a component of the exocyst complex. They share 19% identity and 39% similarity with their amino acid sequences. In addition, it shares around 20% identity with its related proteins

from fruit fly, mouse, rat as well as human. Thus, Sec8p is a well-conserved protein from yeast to human.

3.2.3 Phenotype of *sec8*-1 at the restrictive temperature

sec8-1 cells grew and formed colonies at 24ºC (permissive temperature) but were unable to do so at 36°C (restrictive temperature). To evaluate the phenotype of *sec8*-1 at the restrictive temperature, *sec8*-1 cells were grown at 24ºC to exponential phase and shifted to 36°C. Samples were taken at the shift point (0h) and every hour afterwards (1-4 h) and measured for optical density and cell number. The cell number and optical density of *sec8*-1 did not increase upon temperature shift from 24ºC to 36ºC, whereas wild-type cells continued to grow and divide following an identical temperature shift (Fig 3.2.3A and data not shown).

To further characterize the phenotype of *sec8*-1, changes in the subcellular distribution of F-actin and cell wall materials following shift of *sec8*-1 cells from 24ºC to 36°C (Figure 3.2.3 B) was monitored. *sec8*-1 cells were fixed and stained with DAPI, phalloidin and Calcofluor to visualize DNA, F-actin and cell wall, respectively. Under permissive conditions, F-actin rings and septa in the majority of *sec8*-1 cells resembled those found in wild-type cells (0h). Upon shift to 36°C for four hours, over 50% of *sec8*-1 cells contained four nuclei, indicative of the successful completion of two rounds of mitosis despite the aberrant cytokinesis (4h). Interestingly, under these conditions, assembly and constriction of the actomyosin ring were not impaired in *sec8*-1 cells (arrow). In addition, *sec8*-1 cells were also capable of assembling medial division septa. However, the division septa apparently could not be disassembled in *sec8*-1 cells leading to the accumulation of cells with one or three septa. Thus, *sec8*-1

identifies a protein essential for cell separation following assembly of the division septum.

3.2.4 *sec8*-1 is defective in cell separation, but not in other aspects of cytokinesis

The exocyst is required for polarized cell growth and cell surface expansion in *Saccharomyces cerevisiae* (Roth et al., 1998; TerBush et al., 1996). In contrast, the *sec8*-1 mutant described in this study was defective only in septum disassembly and cell separation, but apparently not impaired with regard to cell surface expansion and polarized growth. To test the role of Sec8p in cell elongation, synchronous populations of wild type and *sec8*-1 cells were monitored for the ability to undergo polarized cell growth and septum assembly. Cells were arrested in the G1 phase of the mitotic cell cycle by nitrogen starvation, and were then transferred into rich medium at 36ºC to allow the resumption of cell growth and division in a synchronous manner. Nitrogen starvation and release provides a convenient means to assess the function of a protein in cell elongation, since cells start at a length of 4 μm and elongate to 14 μm before division (Fig 3.2.4A and B, 0h). After release into rich medium, *sec8*-1 cells were able to elongate, enter mitosis, and assemble division septa with kinetics similar to that of wild-type cells. They were also capable of assembling the actomyosin ring and constricting the ring with a similar kinetics to wild-type cells (data not shown). However, *sec8*-1 cells failed to disassemble the division septa, leading to the accumulation of binucleate cells with a medial division septum, whereas wild-type cells underwent normal cell separation. Although septum cleavage and cell separation failed in *sec8*-1 cells, these cells reinitiated polarized growth and underwent a second round of mitosis and division septum assembly seven hours after release into rich medium. Septum cleavage again failed in these cells resulting in the accumulation of

tetranucleate cells with three septa. Upon prolonged incubation (12 hours) *sec8*-1 cells lysed. The lack of a detectable effect on cell elongation and septum assembly following temperature shift of a synchronous culture of *sec8*-1 established that *sec8*-1 was specifically defective in septum cleavage and cell separation, but not in cell growth and septum assembly.

3.2.5 Identification of *sec6*$^+$, *sec10*$^+$, *sec15*$^+$ and *exo70*$^+$ sequences from the *S. pombe* genome database

The exocyst in *S. cerevisiae* is a multi-protein complex comprising of Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p and Exo84p (TerBush et al., 1996). *S. pombe* Sec8p was identified as a homologue of Sec8p of the exocyst complex in *S. pombe*. I therefore searched the *S. pombe* databases to determine whether other exocyst components were also present in fission yeast. Interestingly, homologues of *S. cerevisiae* Sec6p (*sec6* gene is located in cosmid SPCC1235.10c, accession #: AL031764), Sec10p (SPAC13F5.06c, accession #: Z99091), Sec15p (SPCC1183.01, accession #: AL013740) and Exo70p (SPBC582.02, accession #: AL096788) were also present in *S. pombe*. These *S. pombe* counterparts were referred to as Sec6p, Sec10p, Sec15p and Exo70p in the *S. pombe* database. Proteins related to Sec3p, Sec5p and Exo84p were not identified. The alignments of the *S. pombe* exocyst proteins with their orthologs in other organisms are shown in Fig 3.2.5-1 - Fig 3.2.5-4. These four exocyst proteins in *S. pombe* share ~20% identities in sequences and align through the entire length with their *S. cerevisiae* and mammalian counterparts. Thus, several conserved components of the exocyst complex were identified in *S. pombe*.

3.2.6 The exocyst components interact *in vivo*

3.2.6.1 Sec8p associates with Sec6p, Sec8p and Exo70p

Several exocyst component-like sequences were identified by analysis of *S. pombe* sequences homologous to *S. cerevisiae* sequences. It was therefore interesting to examine whether *S. pombe* Sec6p, Sec8p, Sec10p and Exo70p form a complex *in vivo*, as has been demonstrated for their homologs in other organisms. Immunoprecipitation experiments were performed in order to determine this.

A number of strains expressing either c-Myc or green fluorescent protein (GFP) tagged versions of Sec6p, Sec8p, Sec10p and Exo70p were constructed. The tagged strains resemble wild-type cells and most of these genes are essential for cell viability (refer to Chapter 3.2.9), suggesting that they are likely functional. As shown in Fig 3.2.6.1A-B, Sec6p-GFP, Sec10p-GFP, Sec10p-Myc, Exo70p-Myc were detected specifically from *S. pombe* cell lysates. Genetic crosses were carried out to generate strains *sec8-GFP sec6-Myc*, *sec8-GFP sec10-Myc*, *sec8-GFP exo70-Myc*.

To test the interaction between Sec8p and Sec6p, protein extracts from strains *sec8-GFP*, *sec8-GFP sec6-Myc* and *sec6-Myc* were immunoprecipitated using anti-GFP antibodies. The immune complexes were resolved by SDS-PAGE and probed with a Myc monoclonal antibody to detect Sec6-Myc. The total cell lysates from each strain were also immunoblotted. Sec6p was only detected in immune-complexes generated with GFP antibodies from the *sec8-GFP sec6-Myc* strain, but not in immune-complexes generated from control strains *sec8-GFP* or *sec6-Myc*, suggesting that these two proteins associate *in vivo* (Fig 3.2.6.1C).

Similarly, to test the interaction of Sec8p with Sec10p, immunoprecipitations were performed using protein extracts of strains *sec8-GFP*, *sec8-GFP sec10-Myc*, and *sec10-Myc*. When probing the immune-complexes with anti-Myc antibodies, Sec10p-Myc was detected in immune-complexes obtained from *sec8-GFP sec10-Myc*, but not from that of control strains (Fig 3.2.6.1D). Thus, Sec8p also specifically interacts with Sec10p.

Finally, *sec8-GFP*, *sec8-GFP exo70-Myc*, and *exo70-Myc* strains were constructed to test the interaction between Sec8p and Exo70p. An interaction of Sec8p with Exo70p was demonstrated in similar experiments (Fig 3.2.6.1E).

3.2.6.2 Sec6p, Sec10p and Exo70p associate with each other

To test whether Sec6p was associated with Sec10p, *sec6-myc*, *sec6-myc sec10-GFP* and *sec10-GFP* strains were used to analyze the interaction between Sec6p with Sec10p in a similar way as described in previous sections. Sec6p was detected when Sec10p immune-complexes were precipitated by anti-GFP antibodies (Fig 3.2.6.2A). The anti-myc antibodies did not work well for immunoprecipitation. Hence, it was unable to detect Sec10p-GFP by precipitating complex involving Sec6p-Myc. To confirm the interaction, the tags for Sec6p and Sec10p were swapped by constructing Sec6p-GFP and Sec10p-Myc. Immuno-precipitation was again carried out by anti-GFP antibodies and the complexes were probed with anti-myc antibodies. Sec10p-myc co-immunoprecipitated with Sec6p-GFP, which confirmed that Sec6p interacts with Sec10p (Fig 3.2.6.2B).

In addition, interactions between all exocyst complex members in pair-wise combinations were observed in similar precipitation experiments (Sec6p-Exo70p, and Sec10p-Exo70p, Fig 3.3.2C-D). Our data clearly illustrates that the exocyst components, Sec6p, Sec8p, Sec10p and Exo70p form a complex in *S. pombe*.

3.2.7 Sec6p, Sec8p, Sec10p and Exo70p localize to the division site in *S. pombe*

3.2.7.1 The exocyst components are localized to the division site as well as cell tip(s)

To determine the subcellular localization of the exocyst components, a Sec6p-GFP strain in which the chromosomal copy of the *sec6*$^{+}$ gene was tagged with the GFP epitope was created. In this strain, the expression of Sec6p-GFP was under the control of its native promoter. The Sec6p-GFP cells resembled wild-type cells in morphology and growth rates, indicating that the fusion of Sec6p with GFP is functional. Sec6p-GFP epifluorescence was seen as a faint medial ring structure (Fig 3.2.7.1-1A). Indirect immunofluorescence was therefore performed to confirm the localization of Sec6p-GFP and the same localization pattern was observed as that of Sec6p-GFP epifluorescence (Fig 3.2.7.1-1B). In interphase cells, identified as uninucleate cells with uncondensed chromosomes, tip (s) localization of Sec6p-GFP was observed in 55% of cells. In early mitotic cells, tip (s) localization was diminished and Sec6p-GFP was seen as a ring in the medial region of the cell.

To determine the cell cycle dependent localization of Sec6p, Sec6p-GFP was introduced into a *cdc25*-22 strain (Russell and Nurse, 1986), which allowed cells to arrest at the G2-M transition by a shift to 36°C for 4 hrs. Cells were then released synchronously to the mitotic stage of the cell cycle by growing at 24ºC. Samples were

taken every 15 min and were stained with antibodies against GFP to visualize Sec6p-GFP (Fig 3.2.7.1-2). In G2 cells, Sec6p-GFP did not show any discrete localization. Upon entry into mitosis, Sec6p-GFP was observed as a broad ring at the medial region in metaphase cells with a short spindle. At anaphase, Sec6p-GFP tightened into a ring structure in cells with elongated spindles, and this ring persisted until late anaphase. Upon exiting mitosis, Sec6p-GFP was seen as two adjacent rings in cells with a post-anaphase array of microtubules.

3.2.7.2 The exocyst ring does not undergo constriction

The ring-like localization of Sec6p-GFP was similar in appearance to the actomyosin ring prompted me to examine the localization of exocyst components in relation to the actomyosin ring. Sec10p-GFP (as a marker for the exocyst complex) was introduced into the *cdc25*-22 mutant. The resulting strain was arrested at the G2-M boundary by imposing a four-hour block at 36ºC and then lowering the temperature to 24°C to allow synchronous progression into the cell cycle. Both Sec10p-GFP and actomyosin ring marker Myo2p assembled into ring structures in close proximity at early mitosis (Fig 3.2.7.2). However, constriction of the Sec10p-GFP rings was not observed in those cells where the actomyosin ring underwent constriction in late mitosis (Fig 3.2.7.2). In contrast, Sec10p-GFP rings split into two ring-like structures on either side of the constricting actomyosin ring. Thus, in early mitosis, the exocyst localizes to the division site as a ring structure that at least partially co-localizes with the actomyosin ring. However, while the actomyosin ring undergoes constriction, the exocyst ring does not constrict in late mitosis, but rather splits into two ring/disc-like structures.

3.2.7.3 The exocyst components co-localize in fission yeast

To determine the localization of other exocyst components, Sec8p-GFP, Sec10p-GFP and Exo70p-Myc were examined by immunofluorescence staining by antibodies against GFP or Myc. They all showed similar localization pattern as that of Sec6p-GFP. To determine whether these proteins co-localize, strains *Sec8-GFP Sec6-Myc*, *Sec8-GFP Sec10-Myc* and *Sec8-GFP Exo70p-Myc* were co-stained with antibodies against GFP and Myc to visualize both tagged proteins. As shown in a merged micrograph (Fig 3.2.7.3A), both Sec8p-GFP and Sec6p-Myc co-localized to the cell tip as well as medial ring in the same cells. Thus, Sec8p co-localized with Sec6p.

Colocalization of Sec8p-GFP with Sec10p-Myc and Sec8p-GFP with Exo70p-Myc were also shown in Fig 3.2.7.3B and C. Thus, consistent with co-immunoprecipitation studies, components of the exocyst also colocalized in *S. pombe* cells, which further supported our findings that the exocyst components interact *in vivo*.

3.2.7.4 The exocyst components are localized as two-ring rather than two-disc structure

In cells exiting mitosis, the exocyst was always seen as a two-ring/disc structure at the medial region adjacent to the constricting actomyosin ring. However, it was not clear whether this structure is ring-like, or a disc-like structure. To document this localization, Sec8p-GFP cells were fixed and stained with anti-GFP antibodies to visualize Sec8p-GFP, and serials of scanning micrographs were examined for fluorescence under a Zeiss confocal microscope and analyzed using Z-stack images. These Z-stack micrographs were stacked and rotated to visualize the 3-D structure of Sec8p-GFP. Sec8p-GFP was clearly a two-ring structure, rather than a two-disc structure. This localization was consistent with a potential role in exocytosis, whereby

exocyst components were probably localized at the target plasma membrane to regulate trafficking of secretory vesicles.

3.2.8 The localization of the exocyst components requires F-actin but not secretion

3.2.8.1 The medial localization of the exocyst is dependent on intact F-actin structures

Given that the *S. pombe* exocyst assembled as medial rings, which colocalized with the actomyosin ring at early mitosis, the role of F-actin cytoskeleton and proteins important for actomyosin ring formation in the assembly of the exocyst complex at the site of division was addressed. To investigate whether the medial localization of the exocyst was dependent on F-actin, the localization of Sec6p-GFP after treatment with latrunculin A (LatA), a drug that prevents actin polymerization was monitored. *cdc25*-22 cells expressing GFP-tagged Sec6p were blocked at the G2-M transition point of the cell cycle, and treated either with LatA, or with DMSO as a control upon release from the block into synchronous passage through mitosis. Cells were fixed and stained with antibodies against GFP to visualize Sec6p-GFP. In DMSO treated cells, the F-actin ring was intact and the assembly of medial Sec6p-GFP rings was not affected. However, LatA treated cells in which F-actin was disrupted, the medial Sec6p-GFP rings was not assembled (Fig 3.2.8.1-1), indicating that the proper assembly of Sec6p as a medial ring at the division site is dependent on intact F-actin structures.

To test whether the assembly of other exocyst proteins as a medial ring was dependent on F-actin, Sec10p-GFP was treated with Lat-A in a similar experiment. Again, F-actin is required for the proper assembly of the Sec10p at the medial region of the cell

(Fig 3.2.8.1-2). Thus by inference, the assembly of the exocyst components into a medial ring during cytokinesis requires F-actin.

To ascertain whether the localization of the exocyst was dependent on proteins that are critical for actomyosin ring assembly, Sec10p localization was examined in *cdc8*-110 (Balasubramanian et al., 1992), *cdc12*-112 (Chang et al., 1997) and *cdc15*-140 (Fankhauser et al., 1995) mutants. *cdc8*-110 cells expressing Sec6p-GFP were grown to exponential phase at 24ºC and shifted to 36ºC for 2 and 4 hrs, fixed and stained with anti-GFP and anti-tubulin antibodies to visualize Sec6p-GFP and microtubules, respectively. At 24ºC Sec6p-GFP localized to a medial ring in cells that had mitotic spindles in *cdc8*-110 cells (data not shown). Upon shift to 36ºC, *cdc8*-110 cells that had mitotic spindles were unable to assemble Sec6p-GFP into ring structure (Fig 3.2.8.1-3), suggesting that the assembly of Sec6p to the medial region is dependent on Cdc8p, a protein essential for actomyosin ring assembly.

The localization of Sec6p-GFP in *cdc1*2-112 and *cdc15*-140 mutants was then examined in similar experiments. Sec6p-GFP was absent from these mutants grown at restrictive conditions, indicating that functional Cdc12p (Fig 3.2.8.1-4), an important protein for actomyosin ring assembly, and Cdc15p (Fig 3.2.8.1-5), a protein required for F-actin patch movement to the medial region during cytokinesis, are both required for the assembly of Sec6p-GFP. The localization of Sec10p in *cdc8*-118, *cdc12*-112 and *cdc15*-140 mutants was also examined at the restrictive temperature. Similar finding were observed in these cells (data not shown), indicating that proteins important for actomyosin ring assembly and F-actin dynamics were required for the proper assembly of the exocyst proteins into a medial ring at the division site.

3.2.8.2 The medial localization of the exocyst appears to be independent of exocytosis

Since the exocyst in *S. pombe* is potentially involved in secretion, it was interesting to determine whether the localization of the exocyst as a medial ring was dependent upon the secretory pathway. Brefeldin A (BFA) is a drug that blocks membrane trafficking of newly synthesized proteins from the Endoplasmic Reticulum (ER) to the Golgi apparatus (Turi et al., 1994). Gma12p, a Golgi marker protein, which has been reported to relocate from Golgi to ER upon BFA treatment (Brazer et al., 2000) was included as a control to test the efficacy of BFA treatment. The ER in *S. pombe* is distributed around the nuclear membrane region, while Golgi is seen as patches throughout the cytoplasm (Brazer et al., 2000). *cdc25*-22 cells expressing Sec6p-GFP and *cdc25*-22 cells expressing Gma12-GFP were arrested at the G2-M boundary by growing cultures at 36°C. These cultures were exposed to either BFA or ethanol upon release into mitosis synchronously at 24°C. Cell samples were fixed and stained with antibodies against GFP to visualize Sec6p-GFP or Gma12-GFP. Cps1p, known to be sensitive to the treatment of BFA, was used as an additional control protein. Upon treatment of BFA, Gma12p relocated from Golgi to ER and Cps1p was absent as expected. In contrast, the localization of Sec6p was not affected by BFA (Fig 3.2.8.2-1), indicating that the Sec6p localization is not sensitive to BFA treatment.

To further investigate whether the localization of Sec6p required functional Sec8p, Sec6p localization was examined in *sec8*-1, a mutant defective in exocytosis (see Chapter 3.2.11). At 36°C, Sec6p localization to the division site was not affected in *sec8*-1 cells (Fig 3.2.8.2-2), suggesting that Sec6p localization did not required Sec8p-

dependent exocytosis. Further support for this came from the finding that Sec8p localization was not affected in *rho3Δ* **(**see Chapter 3.2.11**)**, another mutant defective in exocytosis at higher temperatures (see Chapter IV), reaffirming that the localization of Sec6p to the division site does not require both BFA-dependent and exocyst/Rho3p-dependent exocytosis.

In *S. cerevisiae*, the localization of exocyst components is dependent on secretion, except for Sec3p, the targeting factor in the exocyst complex. *S. pombe* Sec6p localized to the division site independent of secretion, prompting me to test whether secretion was required for localization of other exocyst components in fission yeast. Sec10p was examined for its localization after the treatment of BFA and its localization was also found to be resistant to treatment with BFA (Fig 3.2.8.2-3). Consistently, Sec10p localization was also not affected in *sec8*-1 (Fig 3.2.8.2-4). Thus, it is likely that in the absence of the targeting factor Sec3p, each exocyst component in fission yeast was able to accumulate at the division site independent of secretion. Thus, the exocyst complex in *S. pombe* could serve as a landmark for the targeting of the exocytic machinery.

3.2.9 Phenotype of exocyst-null mutants

3.2.9.1 Null mutants of all exocyst components show a cell separation phenotype

While exocyst mutants in *S. cerevisiae* are defective in polarized growth, the *sec8*-1 mutant in *S. pombe* was unaffected with respect to polarized growth. Given that the phenotype of *sec8*-1 in *S. pombe* was dramatically different from that of exocyst mutants described in *S. cerevisiae*, it was possible that *sec8*-1 was not defective in all functions of Sec8p, and thus polarized growth was not affected in *sec8*-1 mutant. If

this explanation were true, *sec8*-null mutants would be expected to show a stronger phenotypic defect with respect to cell growth. To test this, the entire ORF of *S. pombe* *sec8*$^{+}$ was replaced with *ura4*$^{+}$. Meiotic products of these strains were analyzed and Sec8p was found to be essential for cell viability.

To characterize the terminal phenotype of *sec8Δ*, diploid cells carrying one copy of *sec8* null marked with *ura4* gene were sporulated and germinated in minimal medium lacking uracil and stained to visualize F-actin, DNA and septa (Fig 3.2.9.1A). *sec8Δ* mutant spores were capable of germination, cell elongation, mitosis, and division septum assembly. Actomyosin ring assembly was also normal in germinated *sec8Δ* cells. Interestingly, whereas the primary division septa in wild-type cells were cleaved to liberate daughter cells (data not shown), at the same growth conditions primary division septa assembled in the germinating *sec8Δ* cells were not cleaved. Cell growth, cell elongation, and mitosis resumed in unseparated *sec8Δ* cells and eventually leading to the accumulation of tetranucleate cells with septa placed between each pair of nuclei.

To investigate the loss-of-function phenotype of *sec6*, *sec10* and *exo70*, each knock out strain was generated by replacing the respective ORF with *ura4* gene. Tetrad analysis indicated that *sec6* and *sec10* were also essential for cell viability, whereas *exo70* gene is nonessential. Spore germination indicated that *sec6*Δ and *sec10*Δ were also defective in cell separation, but not other aspects of cytokinesis, similar to *sec8*Δ (Fig 3.2.9.1 B and C). *exo70*Δ cells showed a defect in cell separation only at higher temperatures (Fig 3.2.9.1 D). *exo70*Δ was crossed to *sec8*-1, and the double mutant was synthetically lethal at 24°C (data not shown), suggesting that Sec8p and Exo70p

may be involved in parallel pathways or are parts of a protein complex involved in cell separation. Thus, all exocyst components from the fission yeast have a function in cell separation.

3.2.9.2 Sec8p is not detected in *sec8*-null mutant

To ensure that the phenotype was not due to carry over of maternal exocyst proteins, I tested whether the maternal Sec8p was present in *sec8*Δ cells. A diploid strain was constructed in which one *sec8* locus was tagged with Myc marked with *leu1*$^+$, and the other *sec8* locus was replaced with *ura4* gene. It was examined whether the maternal protein, in this case, Sec8p-Myc, was present in the germinated *sec8*Δ mutant cells. S*ec8p-Myc* cells (upper panels) marked with *leu1*$^+$ and *sec8*-null cells (lower panels) marked with *ura4*$^+$ were germinated in appropriate medium, respectively, fixed and stained with antibodies against Myc and Mok1p to visualize Sec8p-Myc and the α-glucan synthase Mok1p (Katayama et al., 1999). Whereas Sec8p-Myc localization was clearly observed in germinated Sec8p-Myc cells, a similar localization was not observed in germinated *sec8*-null cells (Fig 3.2.9.2), suggesting that there was no carry-over of maternal Sec8p in *sec8*Δ cells. Mok1p, used as a control, was observed in both cases as expected. As Mok1p is a α-glucan synthase, the presence of Mok1p in the *sec8*Δ also indicated that Sec8p is not required for the targeting of this transmembrane protein, consistent with previous findings that s*ec8* mutants are not involved in targeting cell wall biosynthetic enzymes for septum formation.

3.2.10 The phenotype of *sec8* shut off

To analyze *sec8* loss-of-function phenotype using a different approach, a *sec8* shut-off strain was constructed in which the *sec8* transcription was under the control of the low

strength nmt promoter (*nmt81* promoter) so that *sec8* transcription could be repressed in the presence of thiamin. *sec8* shut-off cells were grown in medium either lacking thiamin (induced conditions) or containing thiamin (repressed conditions) at 30°C for 14 hours, fixed and stained with DAPI, phalloidin and Calcofluor to visualize DNA, F-actin and septum, respectively. In the absence of thiamin, the *sec8* shut-off strain did not show any phenotype and resembled wild type cells (Fig 3.2.10). However, in the presence of thiamin, *sec8* shut-off cells again were defective only in disassembly of division septum, but not in polarized cell growth (Fig 3.2.10). Therefore, the exocyst is essential for septum disassembly and cell separation. Cell elongation and division septum assembly might require reduced levels of exocyst function or might be independent of it.

3.2.11 The exocyst is involved in exocytosis

3.2.11.1 *sec8*-1 mutant cells accumulate secretory vesicles

The exocyst in budding yeast and mammals is involved in membrane trafficking from the Golgi apparatus to the plasma membrane. To test whether the exocyst in *S. pombe* has a role in exocytosis, it was examined whether the targeting and fusion of secretory vesicles with the plasma membrane could occur normally in *sec8*-1 mutant. Wild-type and *sec8*-1 cells were grown at 24°C, and shifted to 36°C for four hours and processed for electron microscopy following permanganate fixation (Armstrong et al., 1993). Ultra-structural analysis of these cells revealed that secretory vesicles (approximately 100 nm in diameter) were observed only rarely in wild-type cells (Fig 3.2.11.1-1). In contrast, a large number of such vesicles were detected in sections prepared from the *sec8*-1 mutant (Fig 3.2.11.1-2). These vesicles in *sec8*-1 were ~100 nm in diameter and were stained intensely after permanganate fixation, therefore most likely

representing post-Golgi secretory vesicles (Armstrong et al., 1993). In *sec8*-1 mutant cells undergoing septum assembly, most of the vesicles were clustered approximately in the vicinity of the septa. These suggested that while targeting of secretory vesicles to the correct location probably occurred in *sec8*-1, the subsequent docking and /or fusion with the plasma membrane failed. During interphase, *sec8-1* cells were also found to accumulate ~100 nm vesicles, indicating that Sec8p might also participate in exocytic events during interphase.

3.2.11.2 *sec8* shut off mutant accumulate secretory vesicles

To further demonstrate the role of the fission yeast exocyst in a late stage of secretion, *sec8* shut-off cells were grown in the presence or the absence of thiamin and subjected to ultra-structural analysis. Interestingly, when *sec8* transcription was turned off, cells also accumulated a large number of ~100 nm vesicles in the cytoplasm, whereas cells at non-repressed conditions resembled wild-type cells (Fig 3.2.11.2). This data further suggests that *sec8* mutants are defective in exocytosis, which may result in the failed delivery of proteins essential for cell separation.

3.2.12 *sec8*-1 secretes less activity of acid phosphatase

To determine the role played by Sec8p in exocytosis using a different approach, the transport of the enzyme acid phosphatase through the *S. pombe* secretory pathway in *sec8-1* cells was monitored. As ~ 40% of acid phosphatase were secreted into the growth medium, the activity of secreted acid phosphatase from wild-type and *sec8*-1 cells was assayed using the culture. Cells were grown at 24°C (0h) followed by a shift to 36°C and samples were taken every 1 hour afterwards and assayed for the activity of secreted acid phosphatase. As shown in Fig 3.2.12**,** wild-type cells at 36°C secreted

acid phosphatase about twice as fast as at 24°C. *sec8-1* cells secreted much less acid phosphatase than wild-type cells at both temperatures. After 4 h, they secreted 67% of the activity of wild-type cells at 24°C and 42% of the activity at 36°C. Thus, *sec8-1* is indeed defective in exocytosis.

3.2.13 The exocyst does not seem to interact with septins in *S. pombe*

Septin, a conserved large protein complex, have been identified in two yeasts and mammalian cells (Field and Kellogg, 1999; Kartmann and Roth, 2001). The mammalian septins interact with the Sec6/8 complex to regulate the membrane trafficking (Hsu et al., 1998). Recently it has been reported that several septins in *S. pombe* are involved in cell separation (Berlin et al., 2003; Tasto et al., 2003). Thus, I investigated whether septins from *S. pombe* could interact with the exocyst. To compare the intracellular localization of a septin with that of an exocyst protein, Spn1p and Sec10p were examined for their localization in the same cells. Spn1-GFP was crossed to Sec10p-Myc, and cells were stained with antibodies against GFP and Myc to double label Spn1-GFP and Sec10p-Myc. In these cells, Spn1-GFP was found to localize to a medial ring and later split into two rings, co-localized with Sec10p, a marker for the exocyst (Fig 3.2.13).

The inter-dependency of Spn1 and exocyst localization was examined. Spn1p localize normally in *sec8*-1 mutant, and localization of Sec8p was undisturbed in *spn1*Δ, indicating that their localization were independent of each other.

Immunoprecipitation experiments were performed to test whether Spn1-GFP was associated with the exocyst. Strains Spn1-GFP, Spn1-GFP Sec10p-Myc and Sec10p-

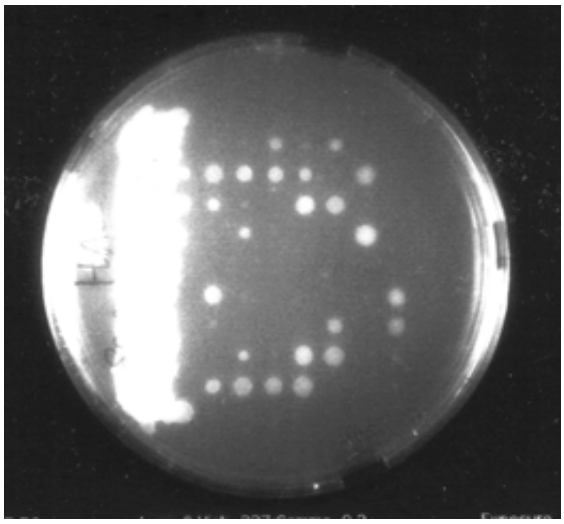
Myc were used for this purpose. However, no interaction could be detected (data not shown). In all, septins does not seem to interact with the exocyst in fission yeast. However, it is possible that other septins not tested here are able to localize the exocyst.

Figure 3.2.1 *mut2*-1 is linked to *sec8* locus. *sec8*-1 (temperature sensitive) was crossed to Sec8-GFP (marked with *ura4*$^{+}$), and the meiotic progenies were dissected on YES. Colonies were then replica plated on either minimal medium lacking uracil and incubated at 24ºC (upper panel), or YES medium and incubated at 36ºC (lower panel) for 1-2 days.

Fig 3.2.1

***sec8*-1 X Sec8-GFP**

ura$^-$, 24°C



YES, 36°C

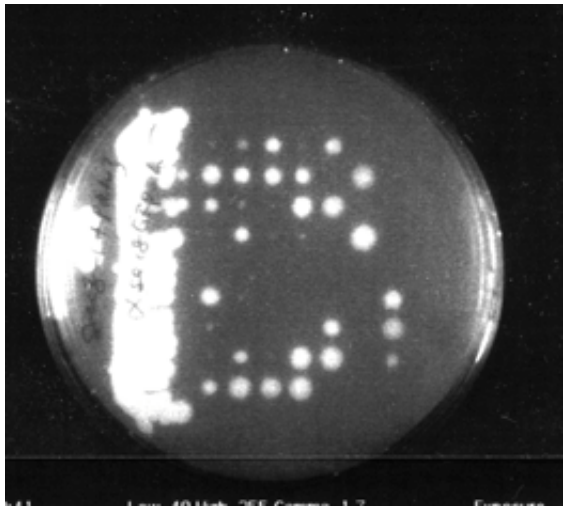


Figure 3.2.2 Alignment of *S. pombe* Sec8p with its related protein from budding yeast and rat. Identical amino acids are shaded in black and conservative substitutions are shaded in grey. *S. pombe* Sec8p share ~20% identity with similar proteins from other organisms.

Fig 3.2.2

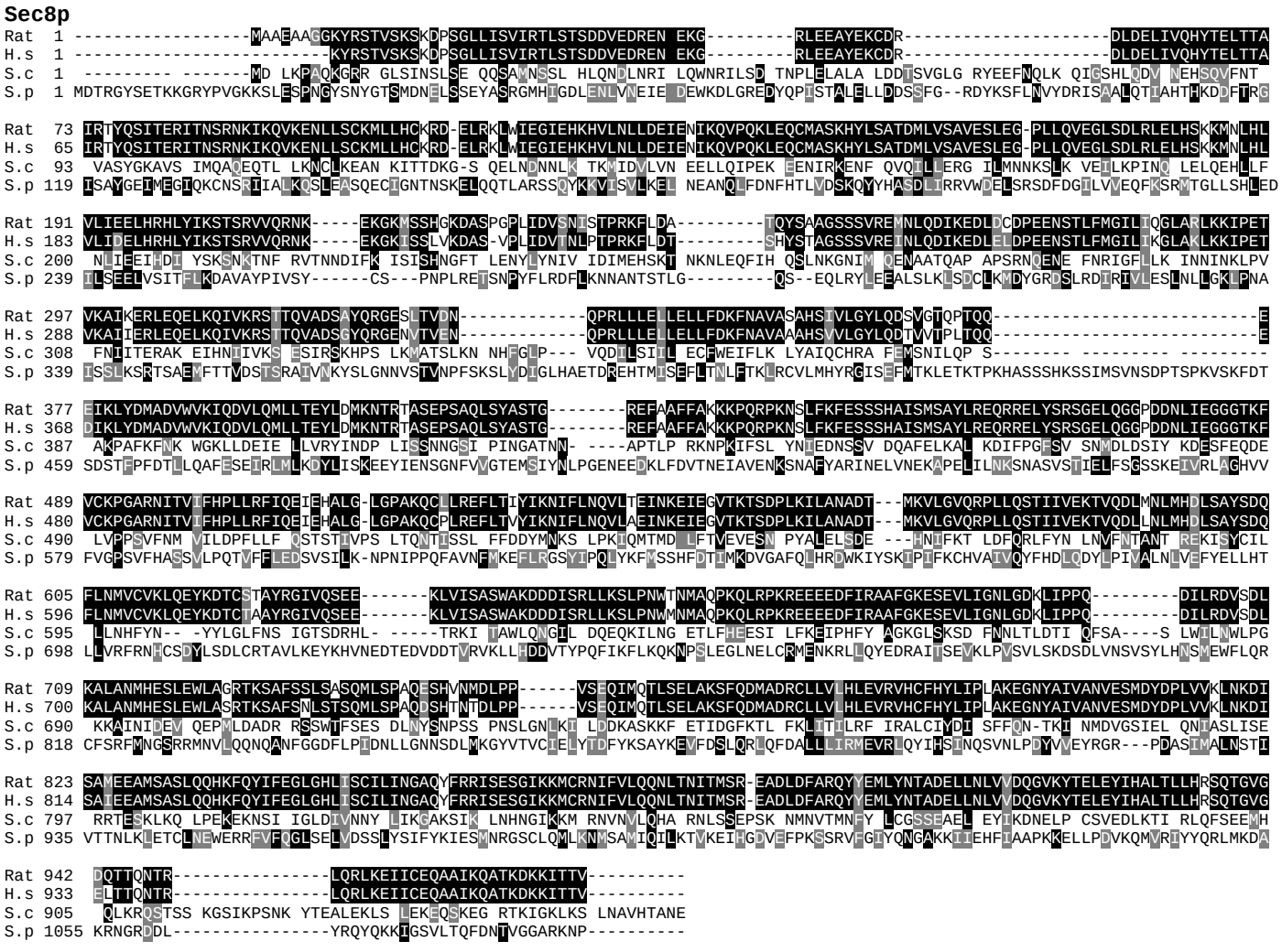
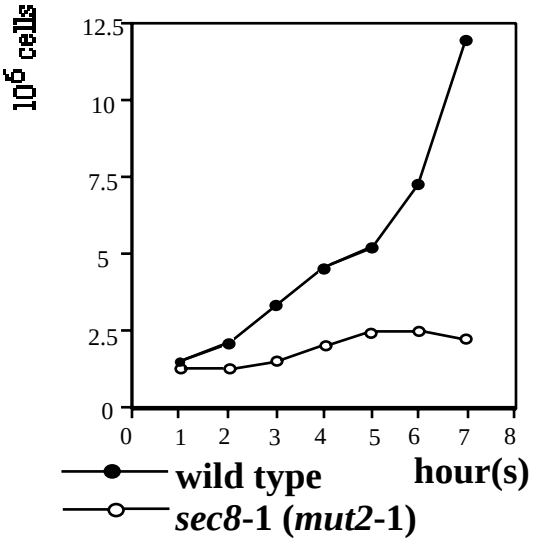


Figure 3.2.3 Phenotype of *sec8*-1 (*mut2*-1) cells. (A) Growth curves of wild-type and *sec8*-1 (*mut2*-1) cells after temperature shift. ●, wild-type; ❍, *sec8*-1 (*mut2*-1). (B) *sec8*-1 (*mut2*-1) is defective in cell separation. *sec8*-1 (*mut2*-1) cells were grown at 24°C and shifted to the restrictive temperature of 36°C for four hours. Samples were collected just prior to the temperature shift (0h), and four hours after the temperature shift and stained with 4',6-diamidino-2-phenylindole (DAPI), rhodamine-conjugated phallioidin and calcofluor to visualize DNA, F-actin and septa, respectively. The constricting actomyosin ring is shown with an arrow.

Fig 3.2.3

A


10^6 cells
12.5
10
7.5
5
2.5
0
0 1 2 3 4 5 6 7 8
hour(s)
wild type
sec8-1 (*mut2*-1)

B

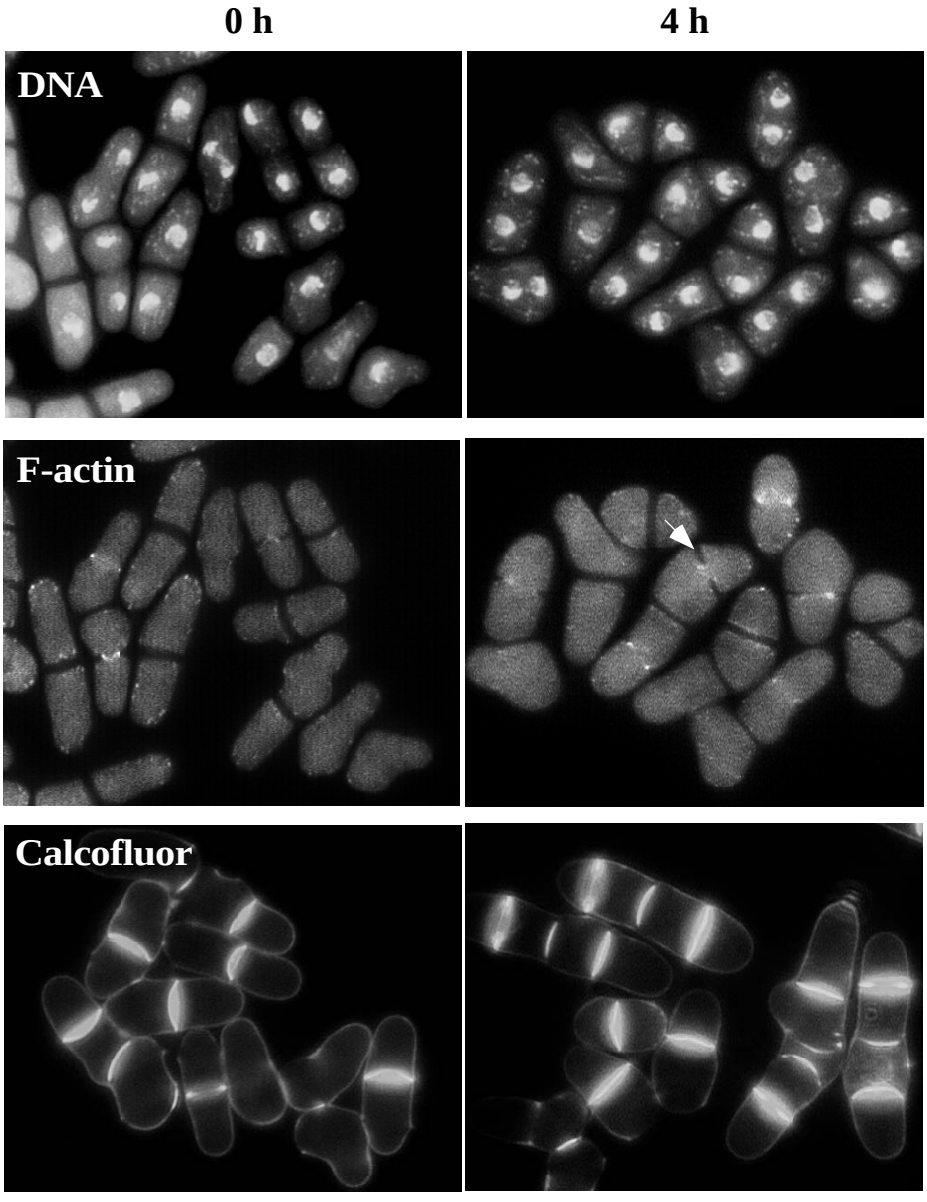
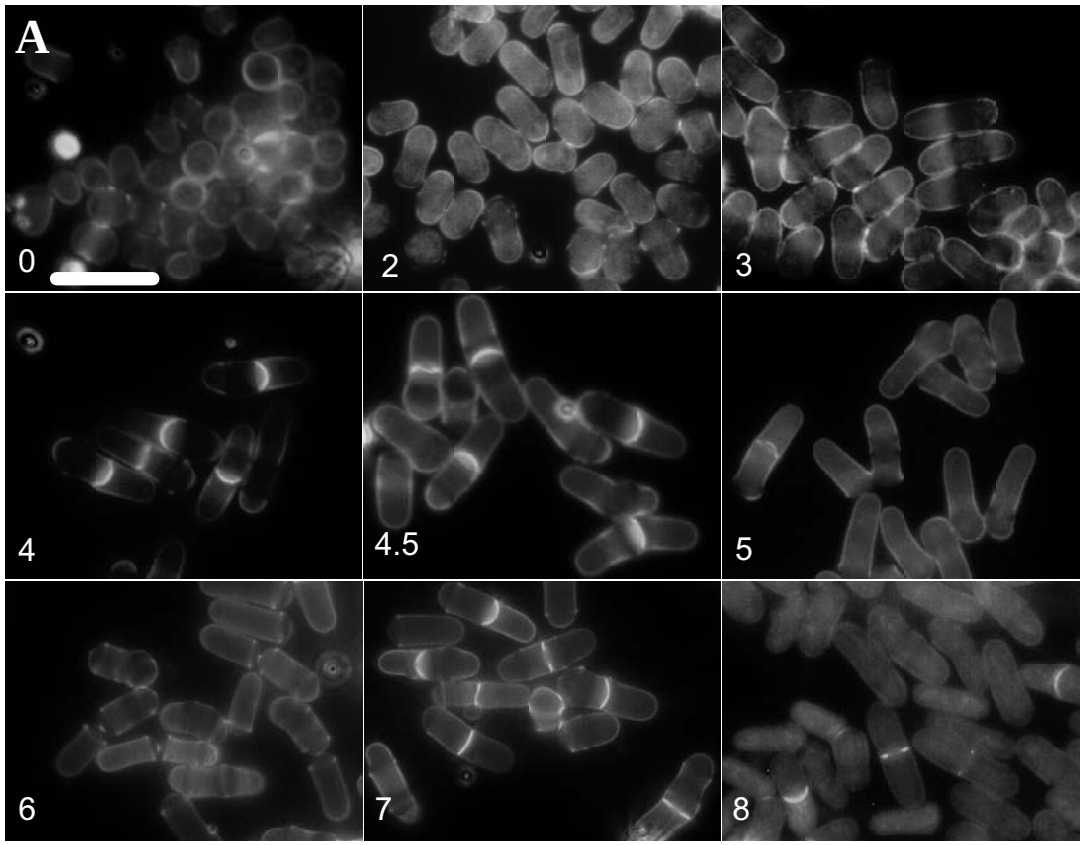

0 h
4 h
DNA
F-actin
Calcofluor

Figure 3.2.4 *sec8*-1 is not defective in polarized cell growth in a synchronous culture. Wild-type cells (A) and *sec8*-1 (B) cells were synchronized in G1 by growth in nitrogen-free medium for 18 hours at 24°C, and shifted to 36°C for one hour to inactivate the Sec8-1 protein. Cells were then resuspended in rich medium to allow cell cycle progression at 36°C. Sample cells were collected just prior to resuspending in rich medium (0h) and every hour after resuspending in rich medium and stained with calcofluor to visualize septa. Bar: 10 μm.

Fig 3.2.4



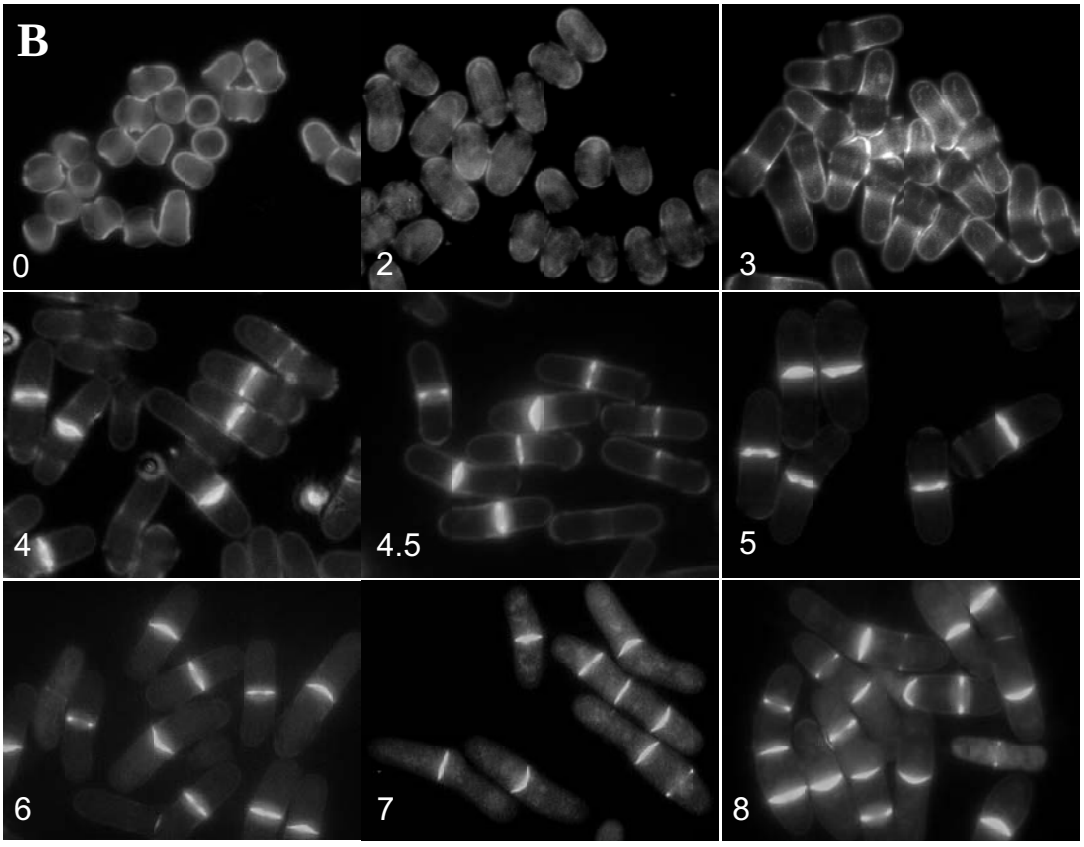


Figure 3.2.5-1 Alignment of *S. pombe* Sec6p with its homologues proteins from budding yeast and rat. Identical amino acids are shaded in black and conservative substitutions are shaded in grey.

Fig 3.2.5-1

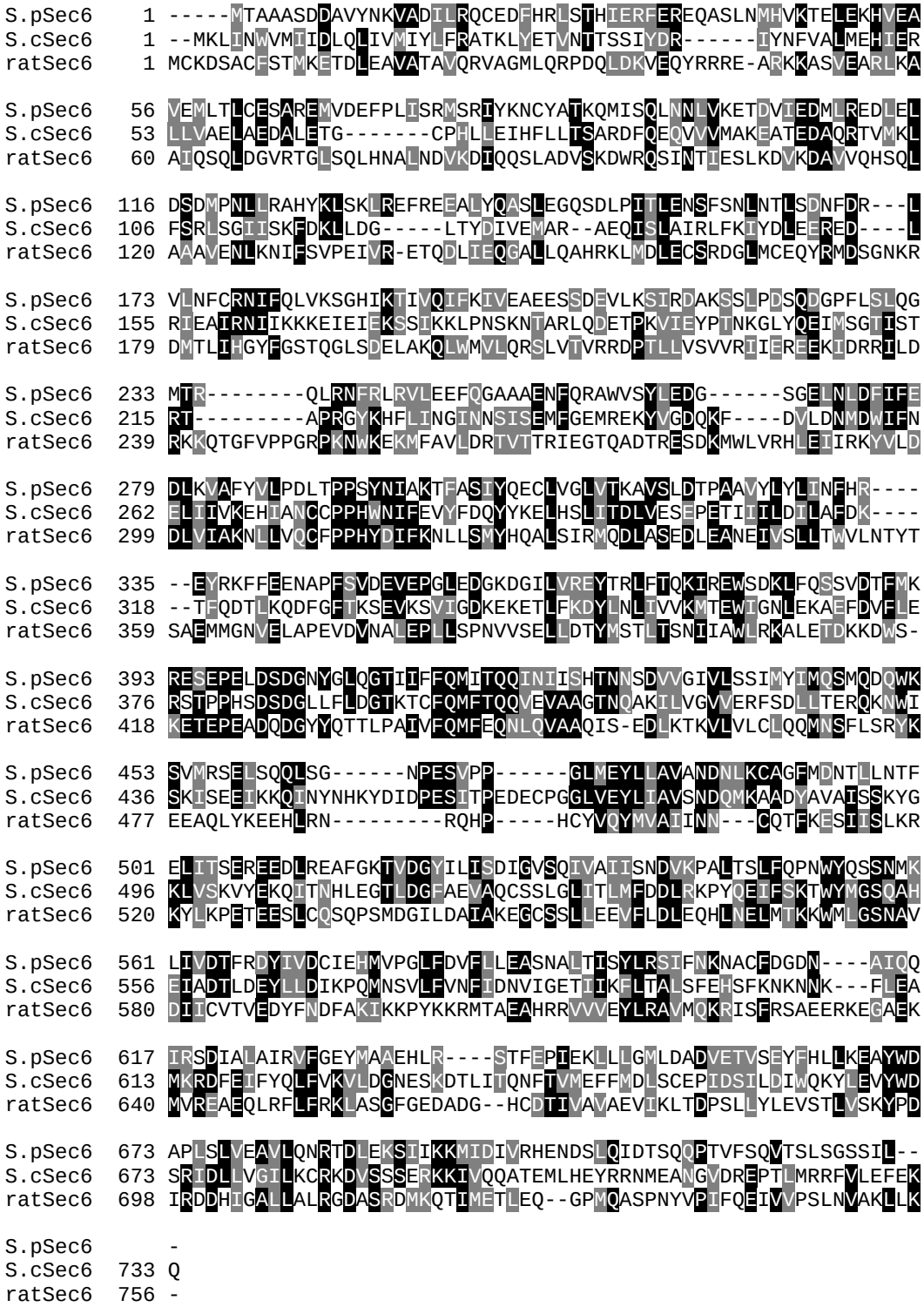

S.pSec6 1 -----MTAAASDDAVYNKVADILRQCEDFHRLSTHIERFEREQASLNMHVKTELEKHVEA
S.cSec6 1 --MKLINWVMIIDLQLIVMIYLFRATKLYETVNITSSIYDR------IYNFVALMEHIER
ratSec6 1 MCKDSACFSTMKETDLEAVATAVQRVAGMLQRPDQLDKVEQYRRRE-ARKKASVEARLKA
S.pSec6 56 VEMLTLCESAREMVDEFPLISRMSRIYKNCYATKQMISQLNNLVKETDVIEDMLREDLEL
S.cSec6 53 LLVAELAEDALETG-------CPHLLEIHFLLTSARDFQEQVVVMAKEATEDAQRTVMKL
ratSec6 60 AIQSQLDGVRTGLSQLHNALNDVKDIQQSLADVSKDWRQSINTIESLKDVKDAVVQHSQL
S.pSec6 116 DSDMPNLLRAHYKLSKLREFREEALYQASLEGQSDLPITLENSFSNLNTLSDNFDR---L
S.cSec6 106 FSRLSGIISKFDKLLDG-----LTYDIVEMAR--AEQISLAIRLFKIYDLEERED----L
ratSec6 120 AAAVENLKNIFSVPEIVR-ETQDLIEQGALLQAHRKLMDLECSRDGLMCEQYRMDSGNKR
S.pSec6 173 VLNFCRNIFQLVKSGHIKTIVQIFKIVEAEESSDEVLKSIRDAKSSLPDSQDGPFLSLQG
S.cSec6 155 RIEAIRNIIKKKEIEIEKSSIKKLPNSKNTARLQDETPKVIEYPTNKGLYQEIMSGTIST
ratSec6 179 DMTLIHGYFGSTQGLSDELAKQLWMVLQRSLVTVRRDPTLLVSVVRIIEREEKIDRRILD
S.pSec6 233 MTR--------QLRNFRLRVLEEFQGAAAENFQRAWVSYLEDG------SGELNLDFIFE
S.cSec6 215 RT---------APRGYKHFLINGINNSISEMFGEMREKYVGDQKF----DVLDNMDWIFN
ratSec6 239 RKKQTGFVPPGRPKNWKEKMFAVLDRTVTTRIEGTQADTRESDKMWLVRHLEIIRKYVLD
S.pSec6 279 DLKVAFYVLPDLTPPSYNIAKTFASIYQECLVGLVTKAVSLDTPAAVYLYLINFHR----
S.cSec6 262 ELIIVKEHIANCCPPHWNIFEVYFDQYYKELHSLITDLVESEPETIIILDILAFDK----
ratSec6 299 DLVIAKNLLVQCFPPHYDIFKNLLSMYHQALSIRMQDLASEDLEANEIVSLLTWVLNTYT
S.pSec6 335 --EYRKFFEENAPFSVDEVEPGLEDGKDGILVREYTRLFTQKIREWSDKLFQSSVDTFMK
S.cSec6 318 --TFQDTLKQDFGFTKSEVKSVIGDKEKETLFKDYLNLIVVKMTEWIGNLEKAFFDVFLE
ratSec6 359 SAEMMGNVELAPEVDVNALEPLLSPNVVSELLDTYMSTLTSNIIAWLRKALETDKKDWS-
S.pSec6 393 RESEPELDSDGNYGLQGTIIFFQMITQQINIISHTNNSDVVGIVLSSIMYIMQSMQDQWK
S.cSec6 376 RSTPPHSDSDGLLFLDGTKTCFQMFTQQVEVAAGTNQAKILVGVVERFSDLLTERQKNWI
ratSec6 418 KETEPEADQDGYYQTTLPAIVFQMFEQNLQVAAQIS-EDLKTKVLVLCLQQMNSFLSRYK
S.pSec6 453 SVMRSELSQQLSG------NPESVPP------GLMEYLLAVANDNLKCAGFMDNTLLNTF
S.cSec6 436 SKISEEIKKQINYNHKYDIDPESITPEDECPGGLVEYLIAVSNDQMKAADYAVAISSKYG
ratSec6 477 EEAQLYKEEHLRN---------RQHP-----HCYVQYMVAIINN---CQTFKESIISLKR
S.pSec6 501 ELITSEREEDLREAFGKTVDGYILISDIGVSQIVAIISNDVKPALTSLFQPNWYQSSNMK
S.cSec6 496 KLVSKVYEKQITNHLEGTLDGFAEVAQCSSLGLITLMFDDLRKPYQEIFSKTWYMGSQAH
ratSec6 520 KYLKPETEESLCQSQPSMDGILDAIAKEGCSSLLEEVFLDLEQHLNELMTKKWMLGSNAV
S.pSec6 561 LIVDTFRDYIVDCIEHMVPGLFDVFLLEASNALTISYLRSIFNKNACFDGDN----AIQQ
S.cSec6 556 EIADTLDEYLLDIKPQMNSVLFVNFIDNVIGETIIKFLTALSFEHSFKNKNNK---FLEA
ratSec6 580 DIICVTVEDYFNDFAKIKKPYKKRMTAEAHRRVVVEYLRAVMQKRISFRSAEERKEGAEK
S.pSec6 617 IRSDIALAIRVFGEYMAAEHLR----STFEPIEKLLLGMLDADVETVSEYFHLLKEAYWD
S.cSec6 613 MKRDFEIFYQLFVKVLDGNESKDTLITQNFTVMEFFMDLSCEPIDSILDIWQKYLEVYWD
ratSec6 640 MVREAEQLRFLFRKLASGFGEDADG--HCDTIVAVAEVIKLTDPSLLYLEVSTLVSKYPD
S.pSec6 673 APLSLVEAVLQNRTDLEKSIIKKMIDIVRHENDSLQIDTSQQPTVFSQVTSLSGSSIL--
S.cSec6 673 SRIDLLVGILKCRKDVSSSERKKIVQQATEMLHEYRRNMEANGVDREPTLMRRFVLEFEK
ratSec6 698 IRDDHIGALLALRGDASRDMKQTIMETLEQ--GPMQASPNYVPIFQEIVVPSLNVAKLLLK
S.pSec6 -
S.cSec6 733 Q
ratSec6 756 -

Figure 3.2.5-2 Alignment of *S. pombe* Sec10p with its homologous proteins from budding yeast and rat. Identical amino acids are shaded in black and conservative substitutions are shaded in grey.

Fig 3.2.5-2

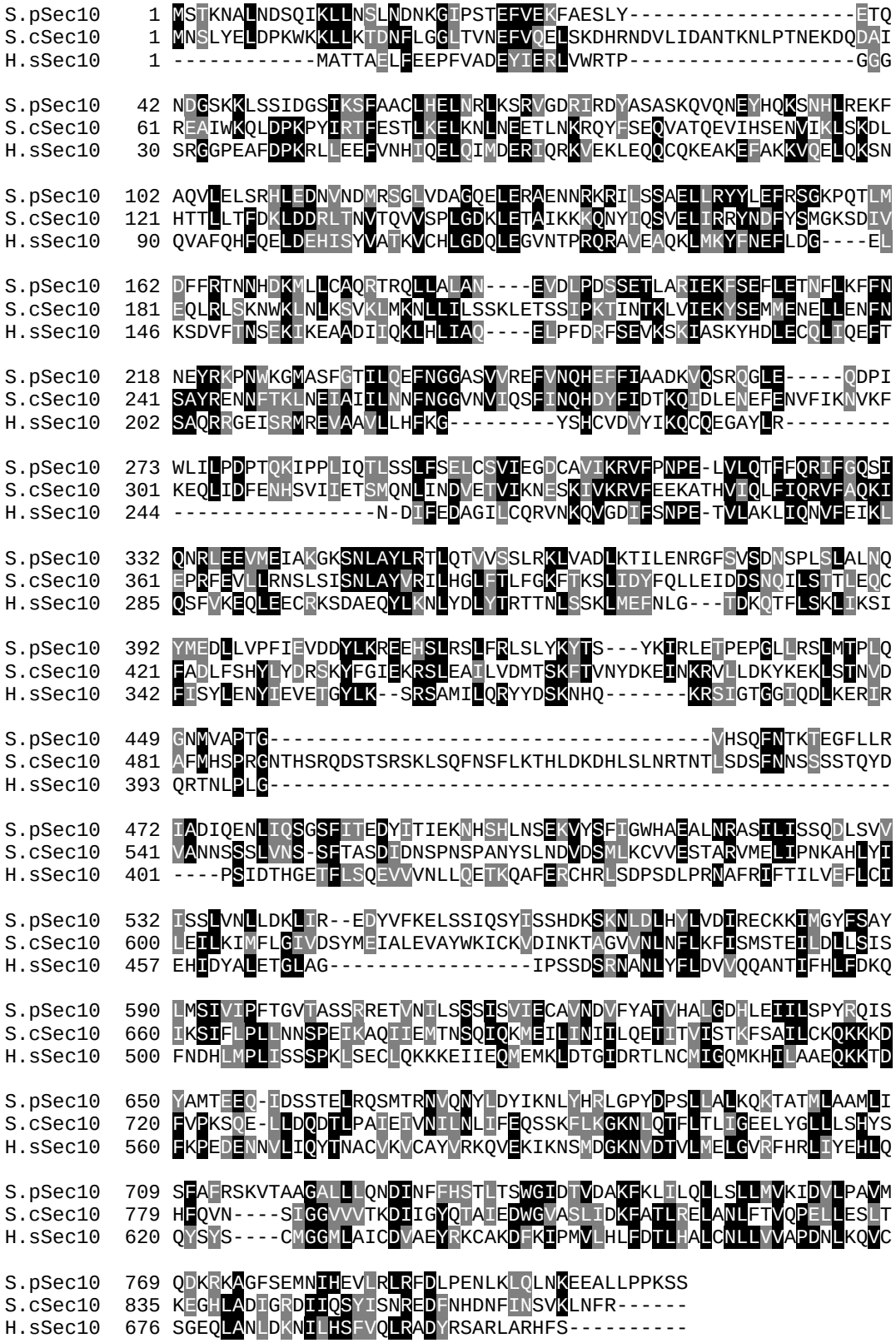


Figure 3.2.5-3 Alignment of *S. pombe* Sec15p with its homologous proteins from budding yeast and rat. Identical amino acids are shaded in black and conservative substitutions are shaded in grey.

Fig 3.2.5-3

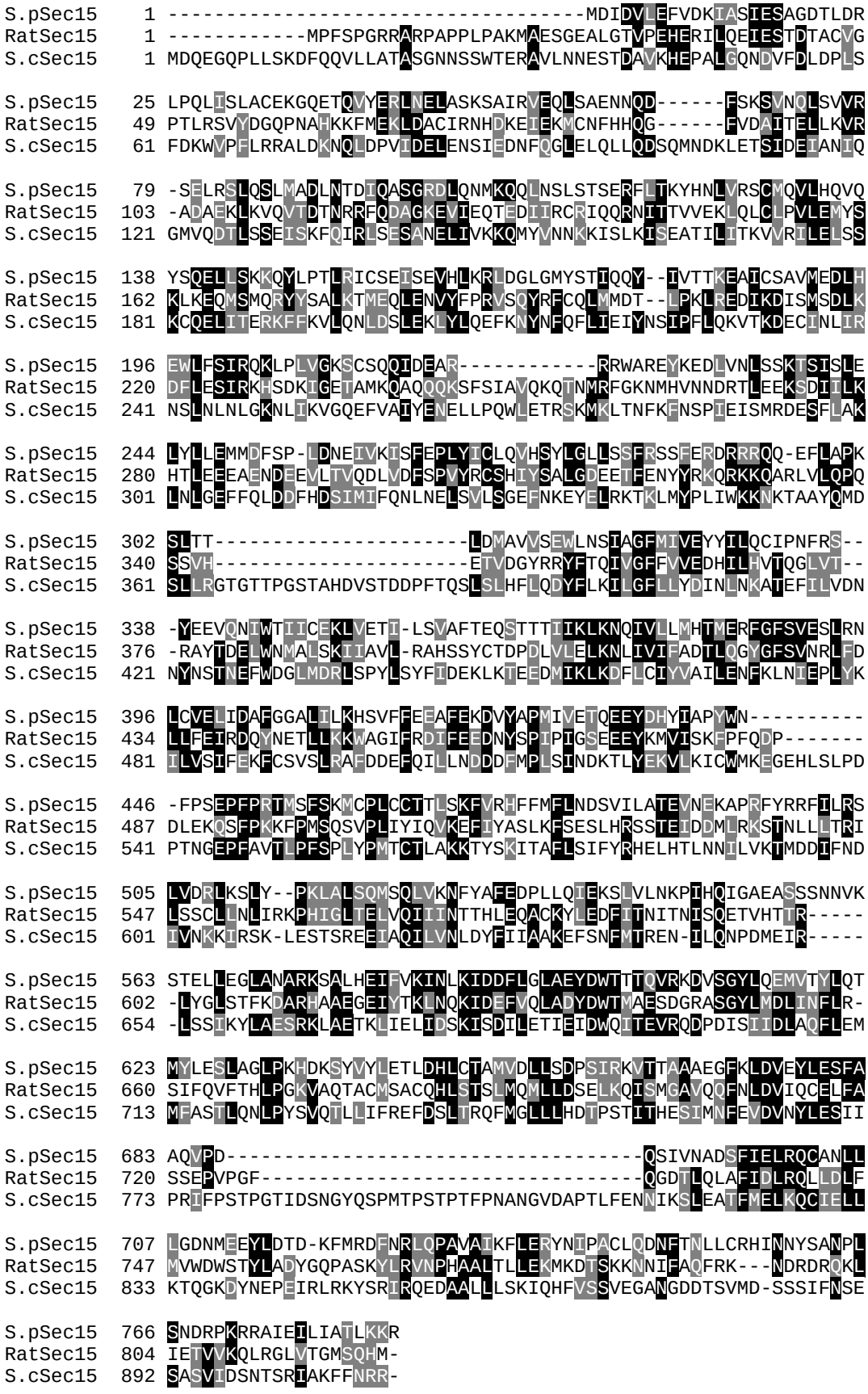

S.pSec15 1 ----------MDIDVLEFVDKIASIESAGDTLDR
RatSec15 1 ----------MPFSPGRRARPAPPLPAKMAESGEALGTVPEHERILQEIESTDTACVG
S.cSec15 1 MDQEGQPLLSKDFQQVLLATASGNNSSWTERAVLNNESTDAVKHEPALGQNDVFDLDPLS
S.pSec15 25 LPQLISLACEKGQETQVYERLNELASKSAIRVEQLSAENNQD------FSKSVNQLSVVR
RatSec15 49 PTLRSVYDGQPNAFKKFMEKLDACIRNHDKEIEKMCNFHHQG------FVDAITELLKVR
S.cSec15 61 FDKWVPFLRRALDKNQLDPVIDELENSIEDNFQGLELQLLQDSQMNDKLETSIDEIANIQ
S.pSec15 79 -SELRSLQSLMADLNTDIQASGRDLQNMKQQLNSLSTSERFLTKYHNLVRSCMQVLHQVQ
RatSec15 103 -ADAEKLKVQVTDTNRRFQDAGKEVIEQTEDIIRCRIQQRNITTVVEKLQLCLPVLEMYS
S.cSec15 121 GMVQDTLSSEISKFQIRLSESANELIVKKQMYVNNKKISLKISEATILITKVVRILELSS
S.pSec15 138 YSQELLSKKQYLPTLRICSEISEVHLKRLDGLGMYSTIQQY--IVTTKEAICSAVMEDLH
RatSec15 162 KLKEQMSMQRYYSALKTMEQLENVYFPRVSQYRFCQLMMDT--LPKLREDIKDISMSDLK
S.cSec15 181 KCQELITERKFFKVLQNLDSLEKLYLQEFKNYNFQFLIEIYNSIPFLQKVTKDECINLIR
S.pSec15 196 EWLFSIRQKLPLVGKSCSQQIDEAR------------RRWAREYKEDLVNLSSKTSISLE
RatSec15 220 DFLESIRKHSDKIGETAMKQAQQQKSFSIAVQKQTNMRFGKNMHVNNDRTLEEKSDIILK
S.cSec15 241 NSLNLNLGKNLIKVGQEFVAIYENELLPQWLETRSKMKLTNFKFNSPIEISMRDESFLAK
S.pSec15 244 LYLLEMMDFSP-LDNEIVKISFEPLYICLQVHSYLGLLSSFRSSFERDRRRQQ-EFLAPK
RatSec15 280 HTLEEEAENDEEVLTVQDLVDFSPVYRCSHIYSALGDEETFENYYRKQRKKQARLVLQPQ
S.cSec15 301 LNLGEFFQLDDFHDSIMIFQNLNELSVLSGEFNKEYELRKTKLMYPLIWKKNKTAAYQMD
S.pSec15 302 SLTT----------LDMAVVSEWLNSIAGFMIVEYYILQCIPNFRS--
RatSec15 340 SSVH----------ETVDGYRRYFTQIVGFFVVEDHILHVTQGLVT--
S.cSec15 361 SLLRGTGTTPGSTAHDVSTDDPFTQSLSLHFLQDYFLKILGFLLYDINLNKATEFILVDN
S.pSec15 338 -YEEVQNIWTIICEKLVETI-LSVAFTEQSTTTIIKLKNQIVLLMHTMERFGFSVESLRN
RatSec15 376 -RAYTDELWNMALSKIIAVL-RAHSSYCTDPDLVLELKNLIVIFADTLQGYGFSVNRLFD
S.cSec15 421 NYNSTNEFWDGLMDRLSPYLSYFIDEKLKTEEDMIKLKDFLCIYVAILENFKLNIEPLYK
S.pSec15 396 LCVELIDAFGGALILKHSVFFEEAFEKDVYAPMIVETQEEYDHYIAPYWN----------
RatSec15 434 LLFEIRDQYNETLLKKWAGIFRDIFEEDNYSPIPIGSEEEYKMVISKFPFQDP-------
S.cSec15 481 ILVSIFEKFCSVSLRAFDDEFQILLNDDDFMPLSINDKTLYEKVLKICWMKEGEHLSLPD
S.pSec15 446 -FPSEPFPRTMSFSKMCPLCCTTLSKFVRHFFMFLNDSVILATEVNEKAPRFYRRFILRS
RatSec15 487 DLEKQSFPKKFPMSQSVPLIYIQVKEFIYASLKFSESLHRSSTEIDDMLRKSTNLLLTRI
S.cSec15 541 PTNGEPFAVTLPFSPLYPMTCTLAKKTYSKITAFLSIFYRHELHTLNNILVKTMDDIFND
S.pSec15 505 LVDRLKSLY--PKLALSQMSQLVKNFYAFEDPLLQIEKSLVLNKPIHQIGAEASSSNNVK
RatSec15 547 LSSCLLNLIRKPHIGLTELVQIIINTTHLEQACKYLEDFITNITNISQETVHTIR-----
S.cSec15 601 IVNKKIRSK-LESTSREEIAQILVNLDYFIIAAKEFSNFMTREN-ILQNPDMEIR-----
S.pSec15 563 STELLEGLANARKSALHEIFVKINLKIDDFLGLAEYDWTTTQVRKDVSGYLQEMVIYLQT
RatSec15 602 -LYGLSTFKDARHAAEGEIYTKLNQKIDEFVQLADYDWTMAESDGRASGYLMDLINFLR-
S.cSec15 654 -LSSIKYLAESRKLAETKLIELIDSKISDILETIEIDWQITEVRQDPDISIIDLAQFLEM
S.pSec15 623 MYLESLAGLPKHDKSYVYLETLDHLCTAMVDLLSDPSIRKVTTAAAEGFKLDVEYLESFA
RatSec15 660 SIFQVFTHLPGKVAQTACMSACQHLSTSLMQMLLDSELKQISMGAVQQFNLDVIQCELFA
S.cSec15 713 MFASTLQNLPYSVQTLLIFREFDSLIRQFMGLLLHDTPSTITHESIMNFEVDVNYLESII
S.pSec15 683 AQVPD----------QSIVNADSFIELRQCANLL
RatSec15 720 SSEPVPGF----------QGDTLQLAFIDLRQLLDLF
S.cSec15 773 PRIFPSTPGTIDSNGYQSPMTPSTPTFPNANGVDAPTLFENNIKSLEATFMELKQCIELL
S.pSec15 707 LGDNMEEYLDTD-KFMRDFNRLQPAVAIKFLERYNIPACLQDNFTNLLCRHINNYSANPL
RatSec15 747 MVWDWSTYLADYGQPASKYLRVNPHAALTLLEKMKDTSKKNNIFAQFRK---NDRDRQKL
S.cSec15 833 KTQGKDYNEPEIRLRKYSRIRQEDAALLLSKIQHFVSSVEGANGDDTSVMD-SSSIFNSE
S.pSec15 766 SNDRPKRRAIEILIATLKKR
RatSec15 804 IETVVKQLRGLVTGMSQHM-
S.cSec15 892 SASVIDSNTSRIAKFFNRR-

Figure 3.2.5-4 Alignment of *S. pombe* Exo70p with its homologous proteins from budding yeast, fly and rat. Identical amino acids are shaded in black and conservative substitutions are shaded in grey.

Fig 3.2.5-4

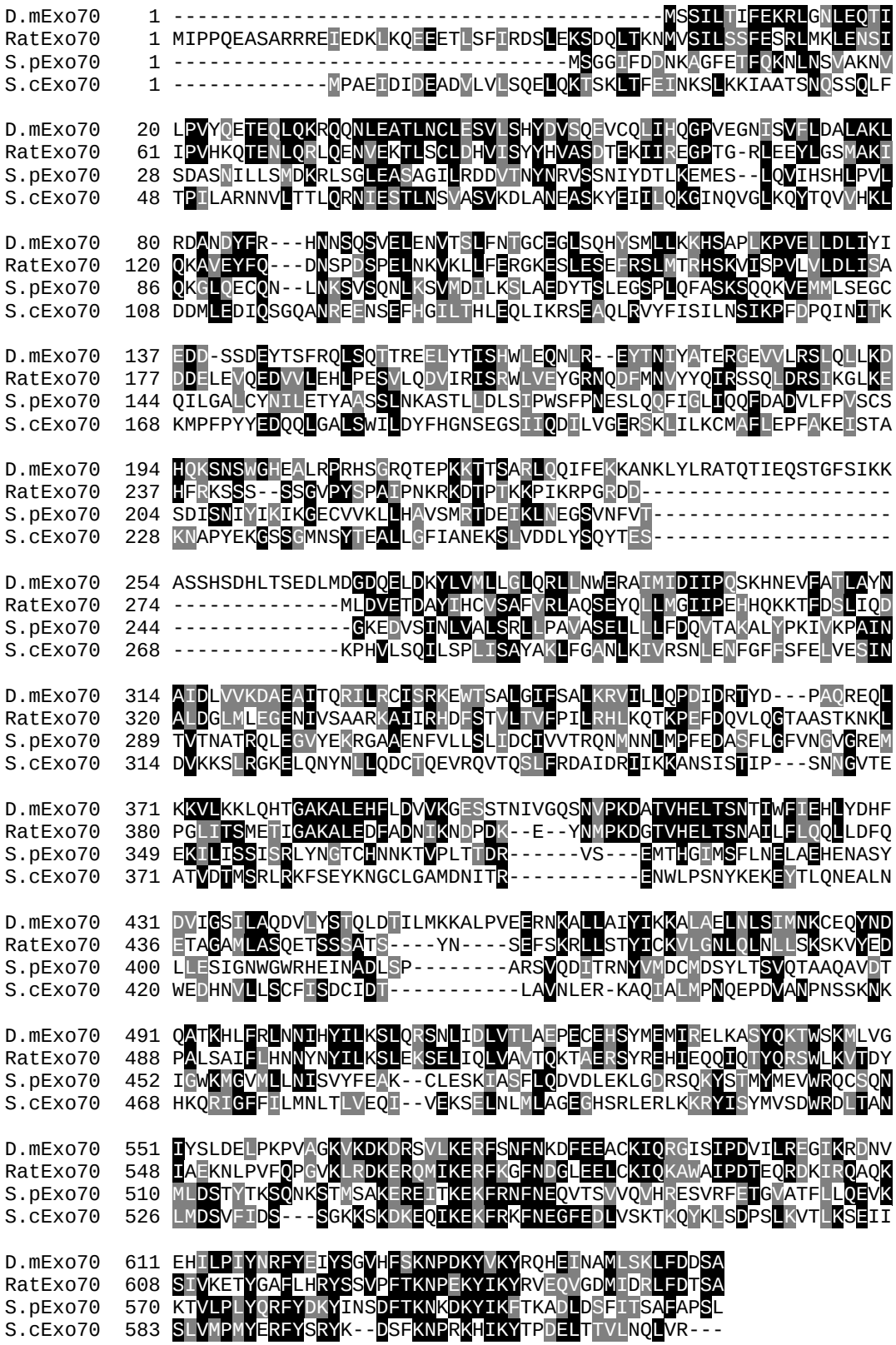
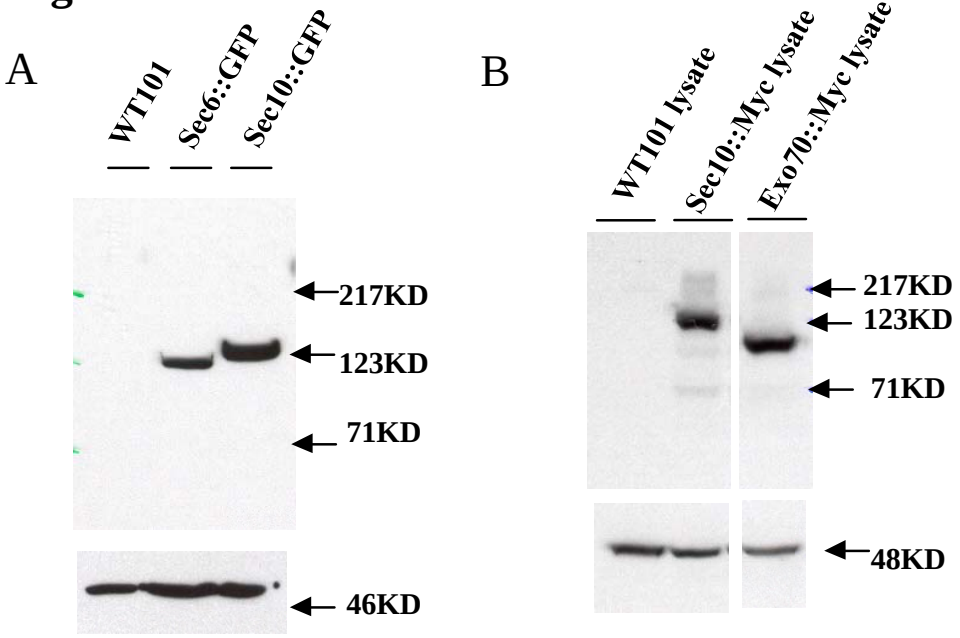


Figure 3.2.6.1 Sec6p, Sec8p, Sec10p and Exo70p associate *in vivo*. (A) Sec6p-GFP, Sec10p-GFP were detected specifically in Western blot. The upper panel was the membrane probed with anti-GFP antibodies. The lower panel was the membrane probed with anti-Arp3 antibodies. (B) Sec10-Myc and Exo70p-Myc were detected specifically in Western blot. The upper panel was the membrane probed with anti-Myc antibodies. The lower panel was the membrane probed with anti-Arp3 antibodies. (C) Co-immunoprecipitation between Sec8p and Sec6p. Protein extracts were prepared from cells expressing Sec8-GFP alone (lane 1), Sec6-Myc and Sec8-GFP (lane 2) and Sec6-Myc alone (lane 3). The upper panel was the membrane probed with anti-Myc antibodies. The lower panel was the membrane probed with anti-GFP antibodies. (D) Co-immunoprecipitation between Sec8p and Sec10p. Protein extracts were prepared from cells expressing Sec8-GFP, Sec10-Myc and Sec8-GFP and Sec10-Myc (E) Co-immunoprecipitation between Sec8p and Exo70p Protein extracts were prepared from cells expressing Sec8-GFP, Exo70-Myc and Sec8-GFP and Exo70-Myc. Immunoprecipitations were performed using anti-GFP antibodies. Immune complexes were separated by SDS-PAGE and analyzed by immuno-blotting using anti-Myc antibodies. 10 μl of each protein extract were loaded as controls.

Fig 3.2.6.1


A
WT101
Sec6::GFP
Sec10::GFP
217KD
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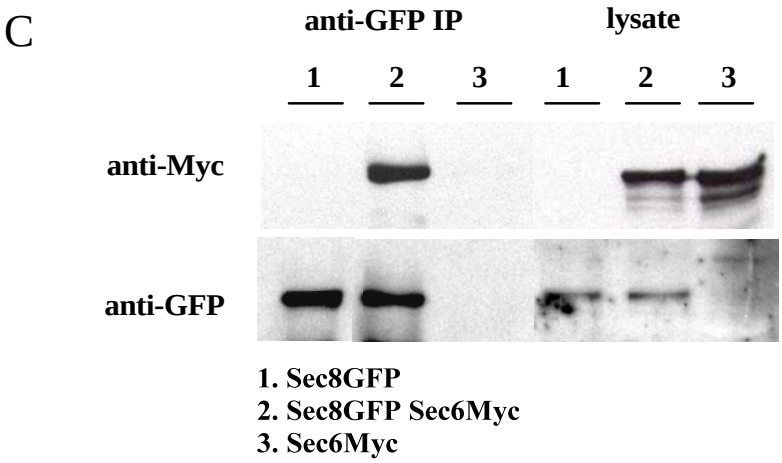
71KD
46KD
B
WT101 lysate
Sec10::Myc lysate
Exo70::Myc lysate
217KD
123KD
71KD
48KD


C
anti-GFP IP
lysate
1 2 3 1 2 3
anti-Myc
anti-GFP
1. Sec8GFP
2. Sec8GFP Sec6Myc
3. Sec6Myc

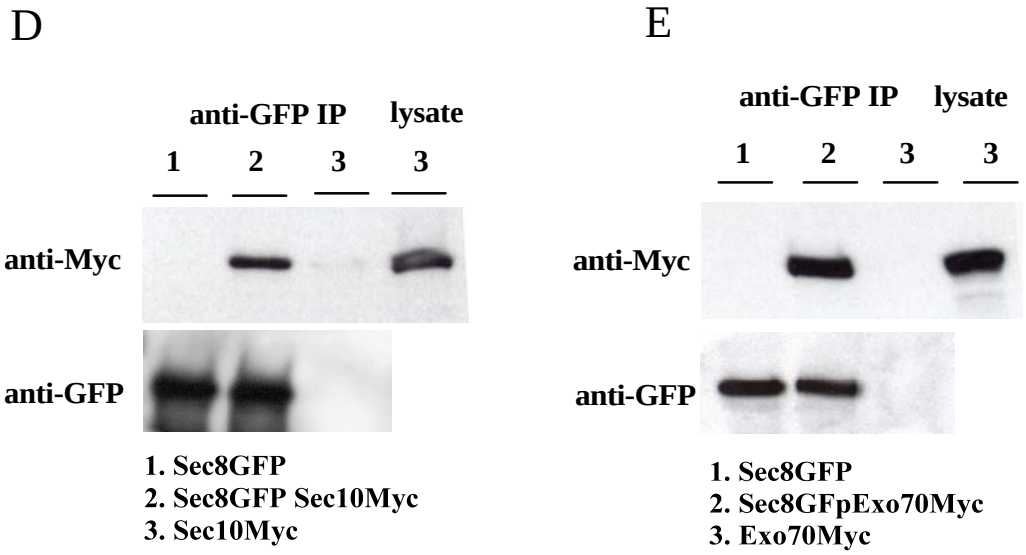
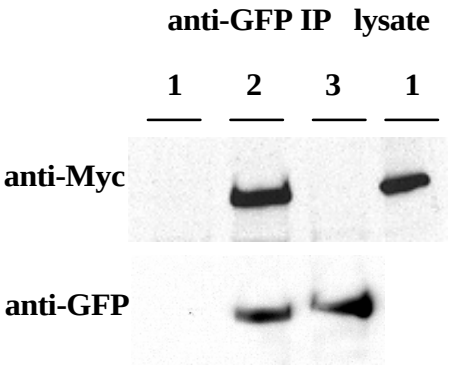

D
anti-GFP IP
lysate
1 2 3 3
anti-Myc
anti-GFP
1. Sec8GFP
2. Sec8GFP Sec10Myc
3. Sec10Myc
E
anti-GFP IP
lysate
1 2 3 3
anti-Myc
anti-GFP
1. Sec8GFP
2. Sec8GFpExo70Myc
3. Exo70Myc

Figure 3.2.6.2 Sec6p, Sec10p and Exo70p associate with each other in vivo. (A) Co-immunoprecipitation between Sec6p and Sec10p. Protein extracts were prepared from cells expressing *sec6-Myc*, *sec6-Myc sec10-GFP* and *sec10-GFP*. (B) Reverse co-immunoprecipitation between Sec6p and Sec10p. Protein extracts were prepared from cells expressing *sec6-GFP*, *sec6-GFP sec10-Myc* and *sec10-Myc*. (C) Co-immunoprecipitation between Sec6p and Exo70p. Protein extracts were prepared from cells expressing *sec6-GFP*, *sec6-GFP Exo70-Myc* and *Exo70-Myc*. (D) Co-immunoprecipitation between Sec10p and Exo70p. Protein extracts were prepared from cells expressing *sec10-GFP*, *sec10-GFP Exo70-Myc* and *Exo70-Myc*. Immunoprecipitations were performed using anti-GFP antibodies. Immune complexes were separated by SDS-PAGE and analyzed by immuno-blotting using anti-Myc antibodies.

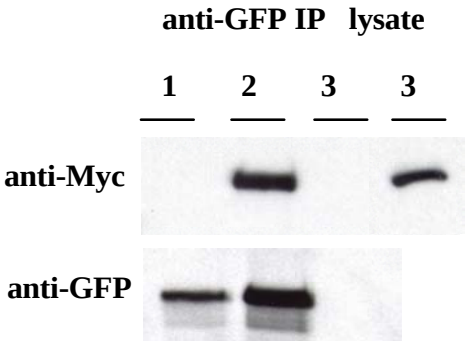
Fig 3.2.6.2

A



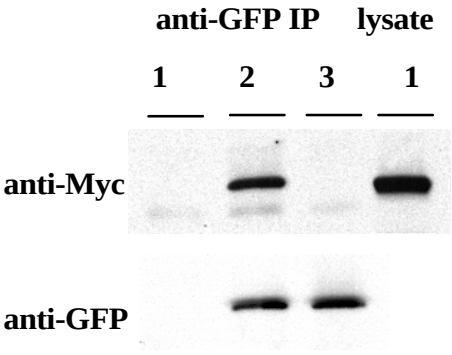
1. Sec6Myc
2. Sec6MycSec10GFP
3. Sec10GFP

B



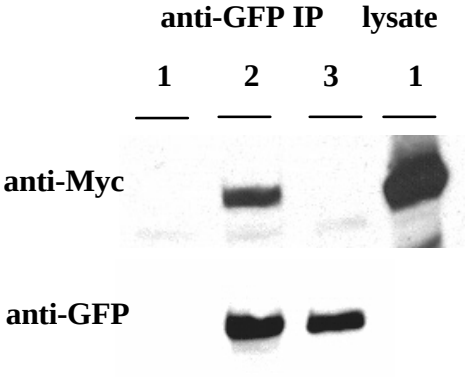
1. Sec6GFP
2. Sec6GFpSec10Myc
3. Sec10Myc

C



1. Exo70Myc
2. Exo70MycSec6GFP
3. Sec6GFP

D



1. Exo70Myc
2. Exo70MycSec10GFP
3. Sec10GFP

Figure 3.2.7.1-1 Localization of the *S. pombe* Sec6p in wild-type cells. (A) epifluorescence of Sec6-GFP. (B) Sec6-GFP in green was detected with anti-GFP antibodies, tubulin was detected with anti-TAT1 antibodies. DNA was stained by DAPI.

Fig 3.2.7.1-1

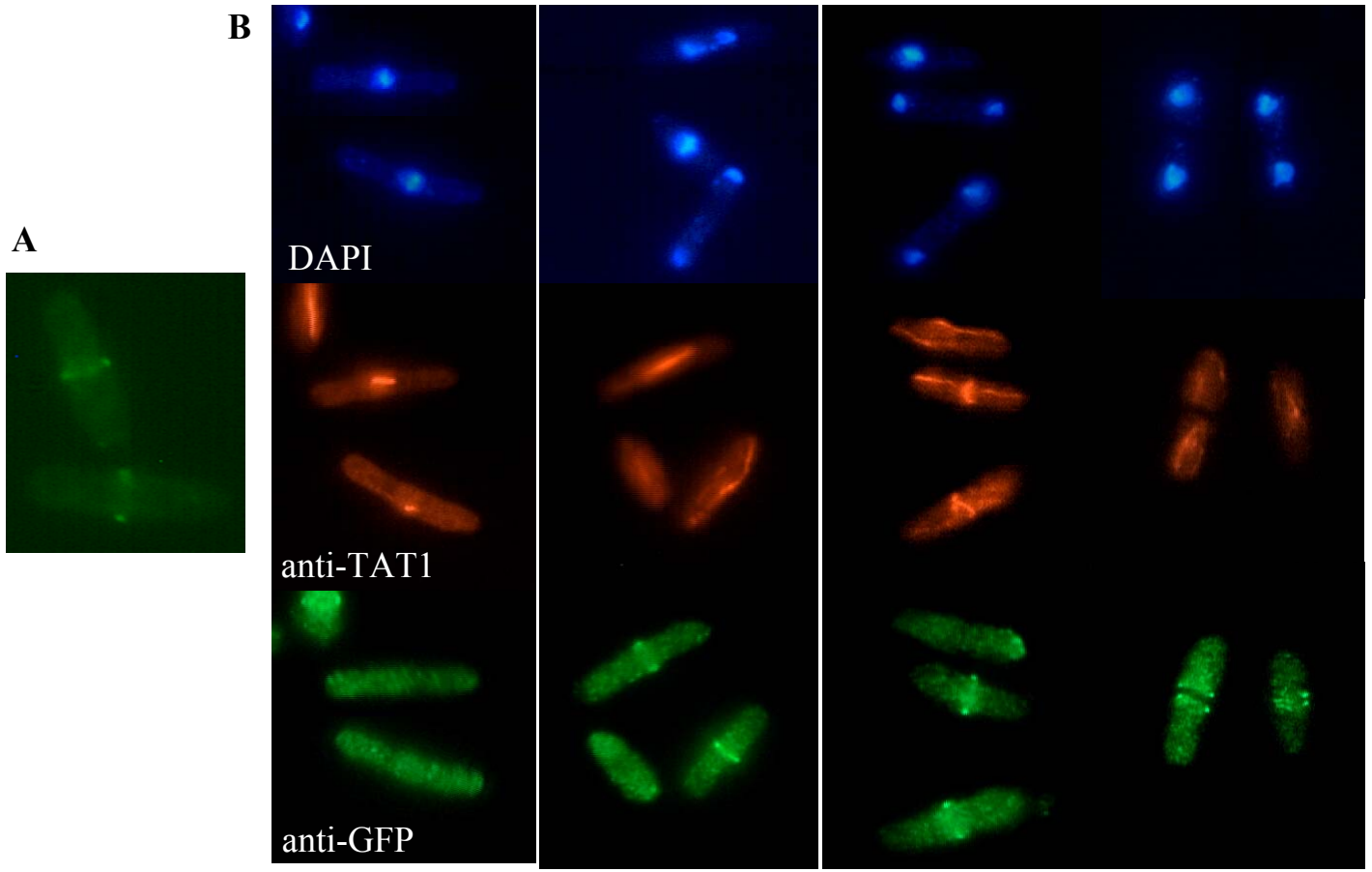


Figure 3.2.7.1-2 Localization of Sec6p in *cdc25*-22 cells. *cdc25*-22 cells expressing Sec6p were blocked at G2-M boundary and released into mitosis synchronously. Samples were taken every 15min after release, and stained to visualize Sec6-GFP in green, DNA in blue and tubulin in red.

Fig 3.2.7.1-2

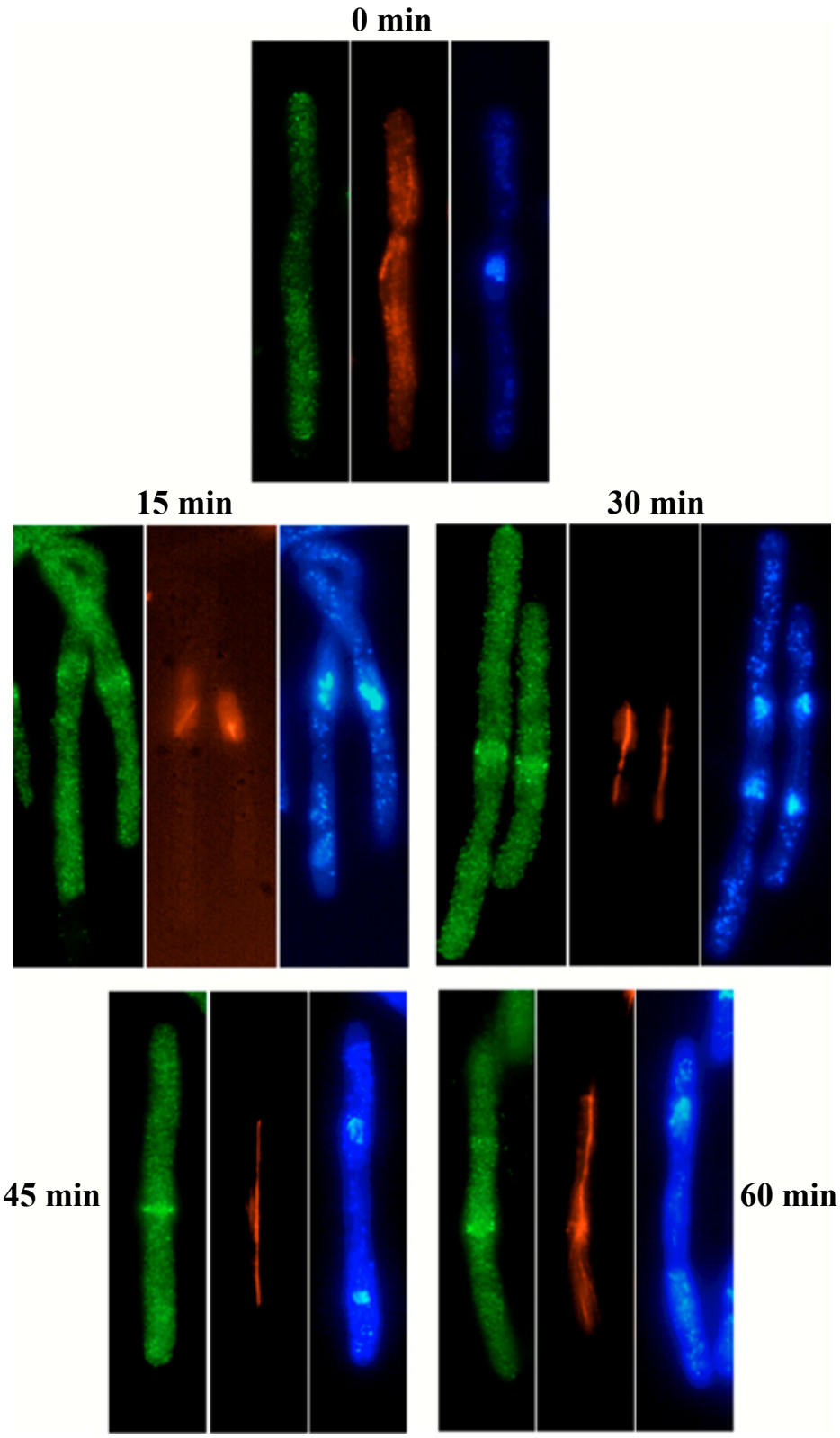
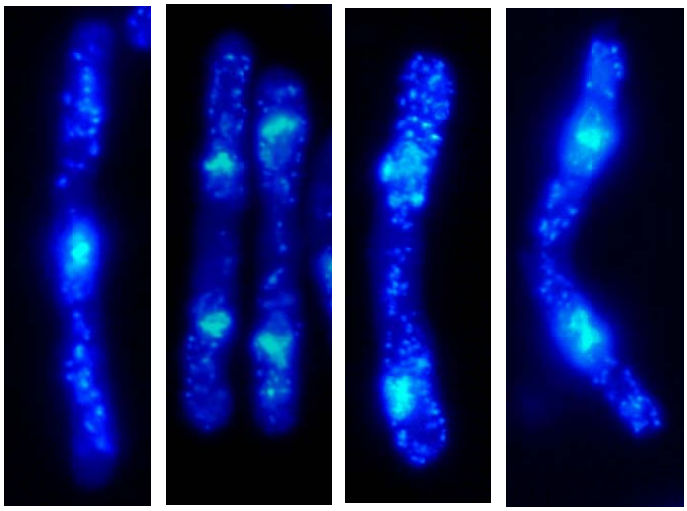


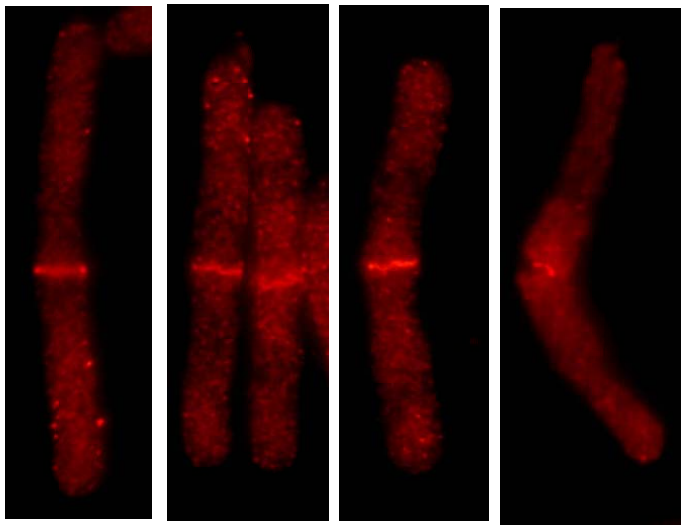
Figure 3.2.7.2 The exocyst ring does not constrict like that observed for the actomyosin ring. *cdc25*-22 cells expressing Sec10-GFP were blocked at G2-M entry and released into mitosis. Samples were taken every 25min and stained to visualize Sec10p in green, Myo2p in red and DNA in blue.

Fig 3.2.7.2

DNA



Myo2



Sec10-GFP

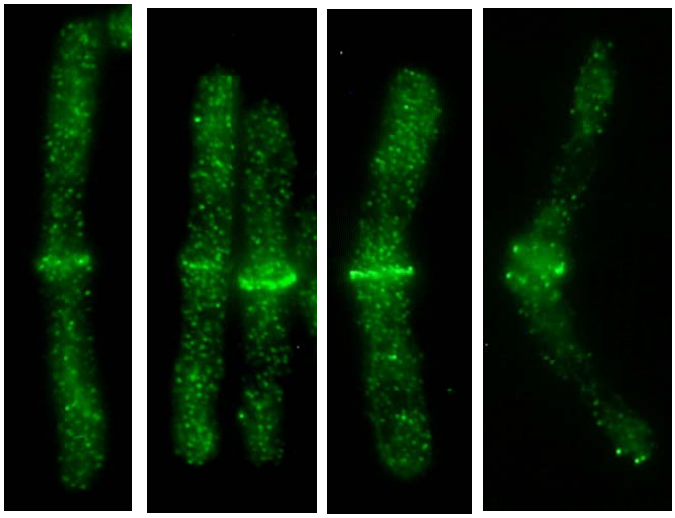


Figure 3.2.7.3 Colocalization of the exocyst proteins. (A) Sec8p colocalizes with Sec6p at the division site as well as cell tip(s). *Sec8-GFP sec6-Myc* cells were stained with antibodies against GFP and Myc. Sec8-GFP is in red, Sec6-Myc in green, and DNA in blue. (B) Colocalization of Sec8p with Sec10p. Sec8-GFP is in red, Sec10-Myc in green, and DNA in blue. (C) Colocalization of Sec8p with Exo70p. Sec8-GFP is in red, Exo70-Myc in green, and DNA in blue.

Fig 3.2.7.3

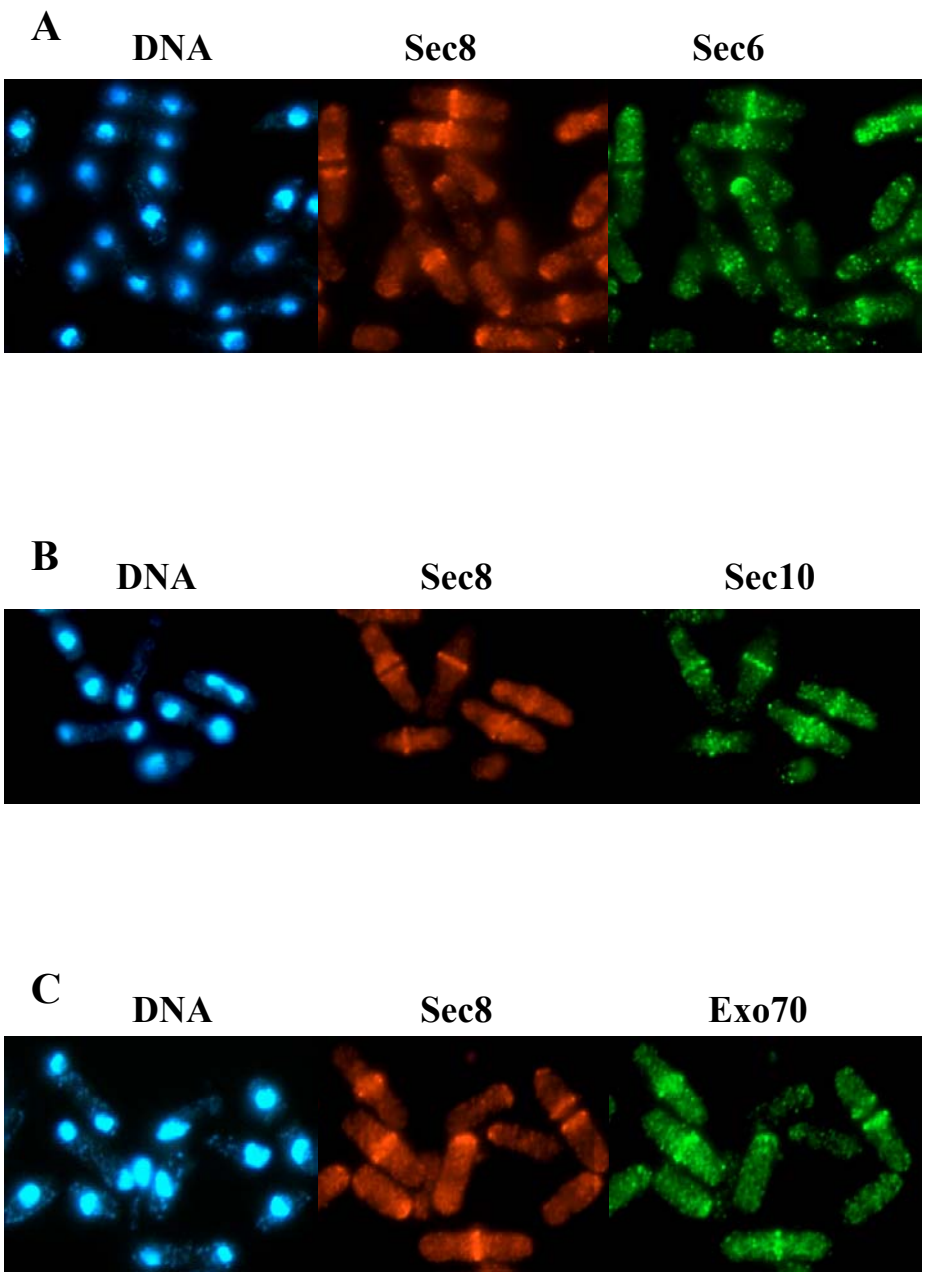
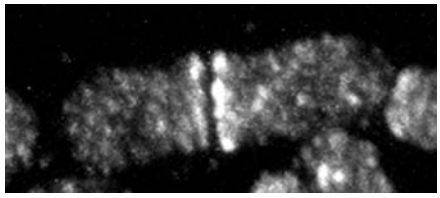


Figure 3.2.7.4 The exocyst localizes to two-ring structure rather than two discs. Sec8-GFP appeared as two rings in late mitotic cells using confocal microscopy and 3D viewing. Top panel, cell with 0° rotation; lower panel, cell with 139° rotation.

Figure 3.2.7.4

0°



139°

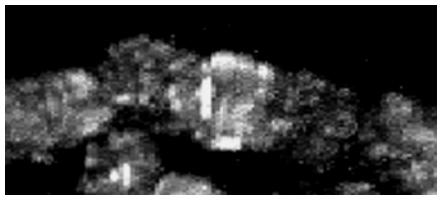


Fig 3.2.8.1-1 The assembly of Sec6p ring at the division site is dependent on F-actin. *cdc25*-22 cells expressing Sec6-GFP were treated with either Lat-A in DMSO (B) or DMSO alone (A) upon release into mitosis. Samples were stained with antibodies against GFP and Tubulin to visualize Sec6-GFP and microtubules.

Fig 3.2.8.1-1

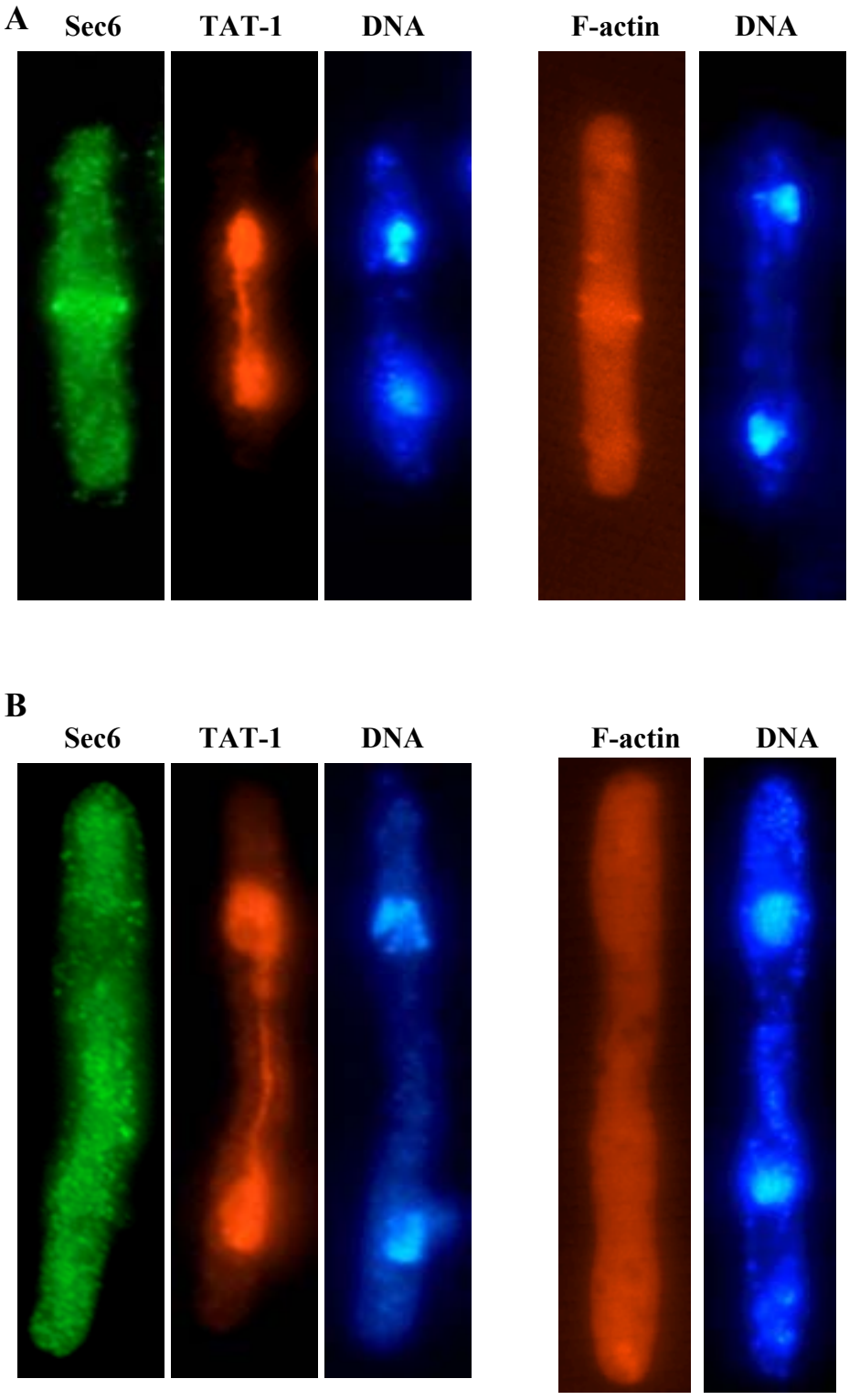


Figure 3.2.8.1-2 The assembly of Sec10p ring requires intact F-actin structures. *cdc25*-22 cells expressing Sec10-GFP were treated with either Lat-A in DMSO (B) or DMSO alone (A) upon release into mitosis. Samples were stained with antibodies against GFP and Tubulin to visualize Sec6-GFP and microtubules.

Fig 3.2.8.1-2

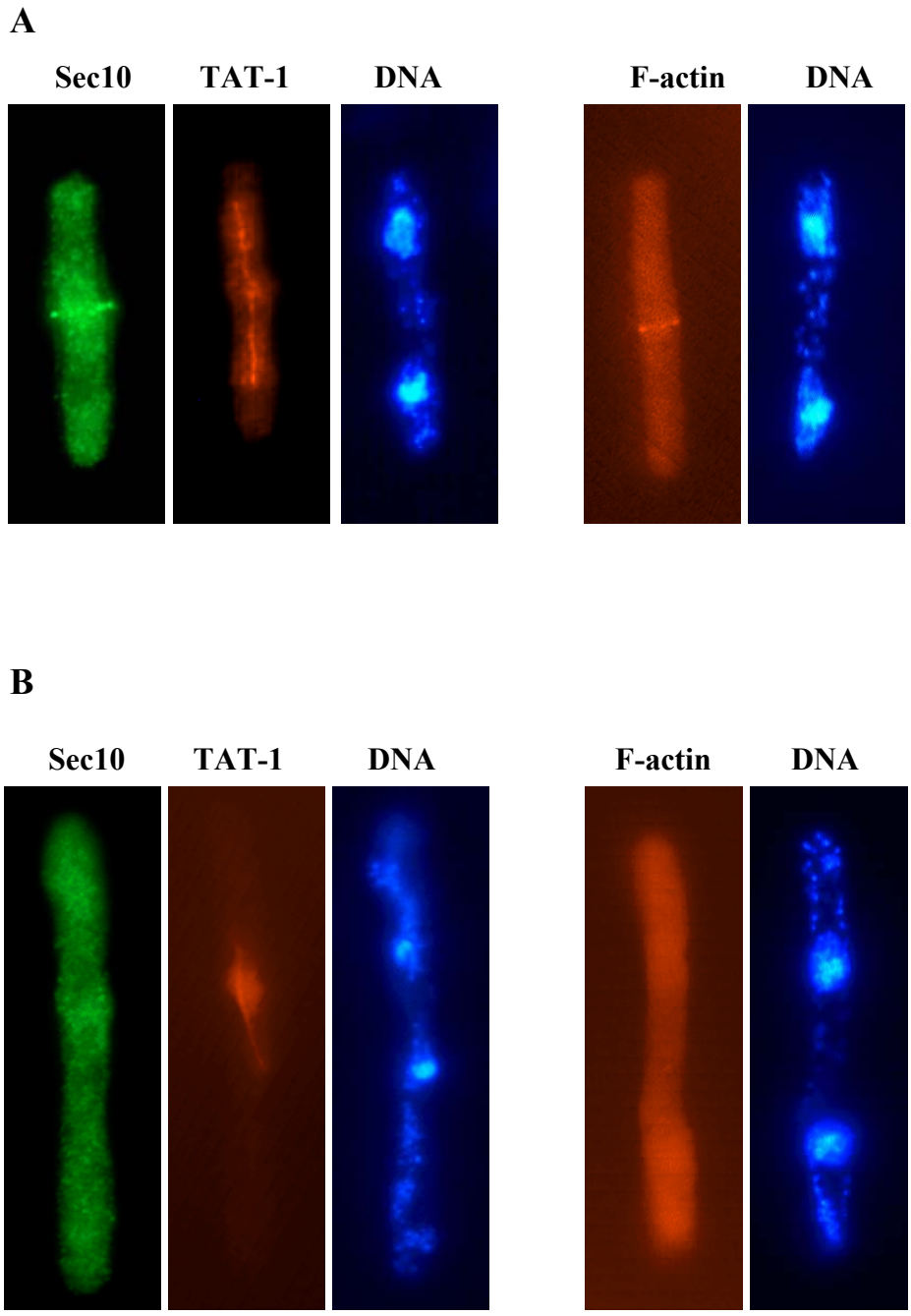


Figure 3.2.8.1-3 The localization of Sec6p is dependent on functional Cdc8p. *cdc8*-110 cells expressing Sec6-GFP were grown at 24°C followed by a shift to 36°C for 2hrs (upper panels) and 4 hrs (lower panels). Samples were stained with antibodies against GFP and Tubulin to visualize Sec6-GFP and microtubules, respectively.

Figure 3.2.8.1-4 The localization of Sec6p is dependent on functional Cdc12p. *cdc12*-112 cells expressing Sec6-GFP were grown at 24°C followed by a shift to 36°C for 2hrs (upper panels) and 4 hrs (upper panels). Samples were stained with antibodies against GFP and Tubulin to visualize Sec6-GFP and microtubules, respectively.

Fig 3.2.8.1-3

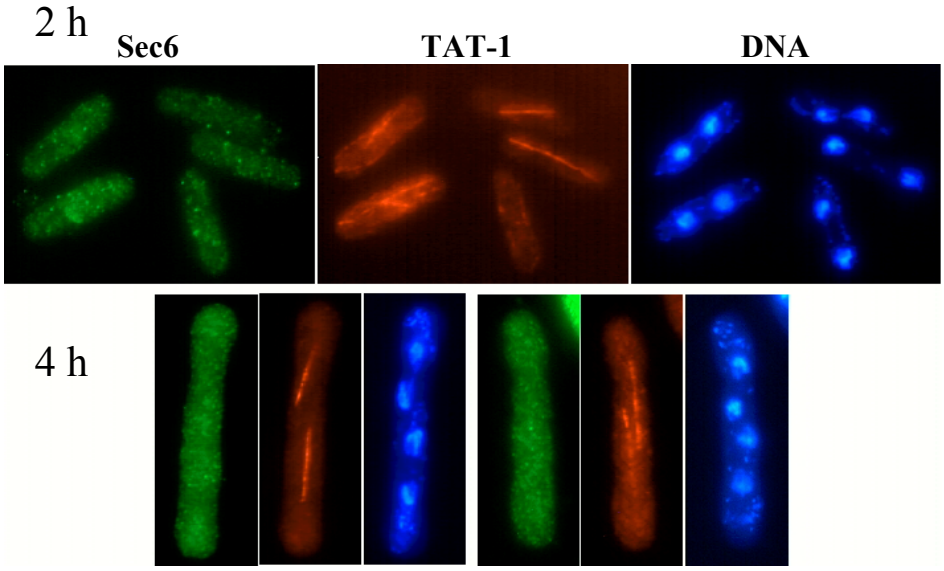


Fig 3.2.8.1-4

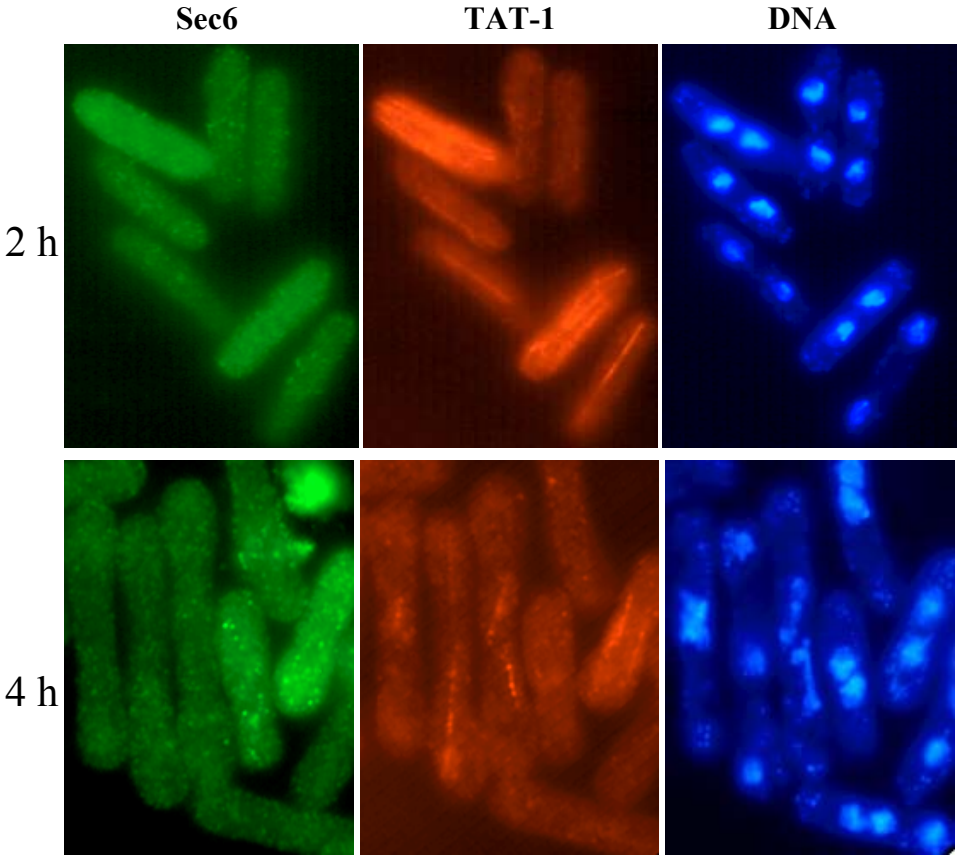


Figure 3.2.8.1-5 The localization of Sec6p requires functional Cdc15p. *cdc15*-140 cells expressing Sec6-GFP were grown at 24°C followed by a shift to 36°C for 4 hrs. Samples were stained with antibodies against GFP and Myo2p to visualize Sec6-GFP and Myo2p, respectively.

Fig 3.2.8.1-5

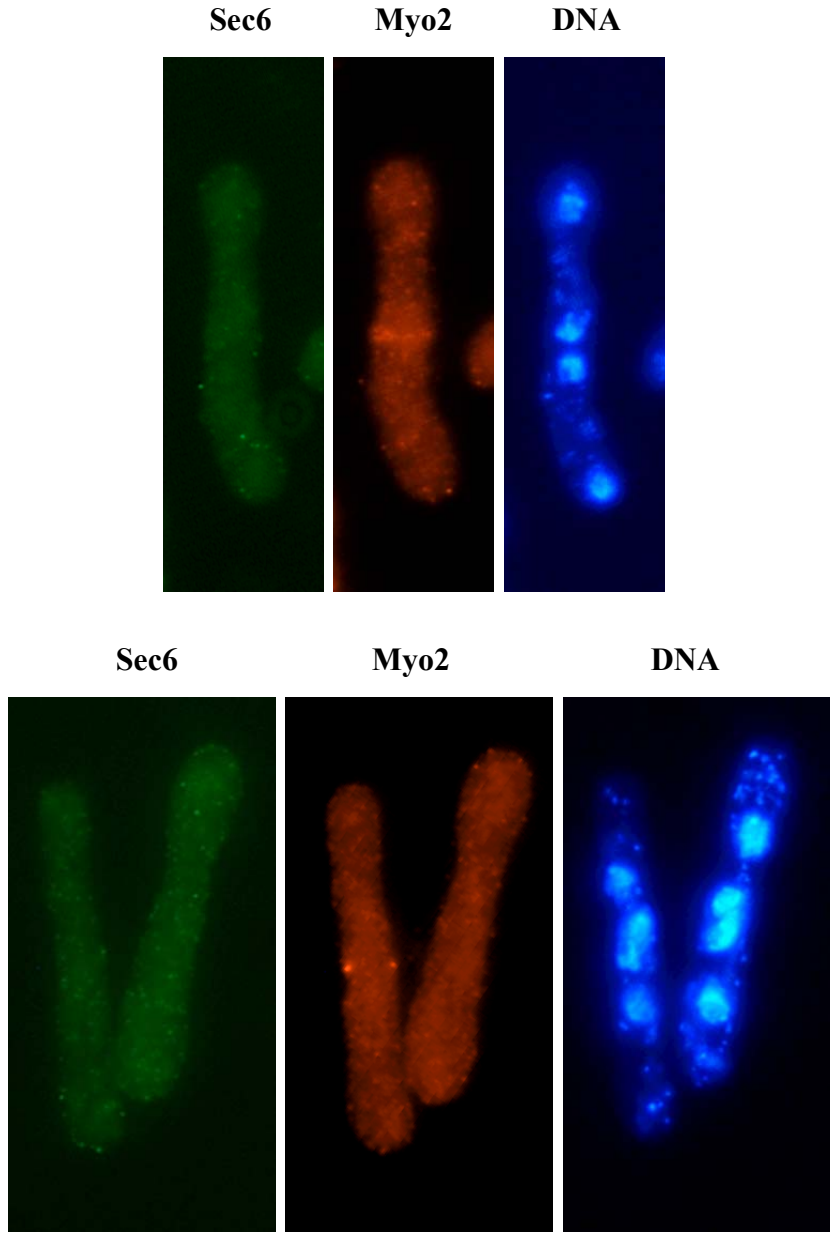


Figure 3.2.8.2-1 The medial localization of Sec6p does not require exocytosis. *cdc25*-22 cells expressing Sec6-GFP were treated with Brefeldin A (left panels), a drug blocking membrane trafficking of newly synthesized proteins from Endoplasmic Reticulum (ER) to Golgi (Turi et al., 1994) or with ethanol (right panels) as a control. Samples were stained with antibodies against GFP and Tubulin to visualize Sec6-GFP and microtubules (A). Samples cells were also stained with anti-Drc1 antibodies to visualize Drc1p (B), whose localization is known to be disrupted by BFA. *cdc25*-22 cells expressing Gma12-GFP (C), served as another control were treated with BFA and examined for Gma12 localization by staining with anti-GFP antibodies.

Fig 3.2.8.2-1

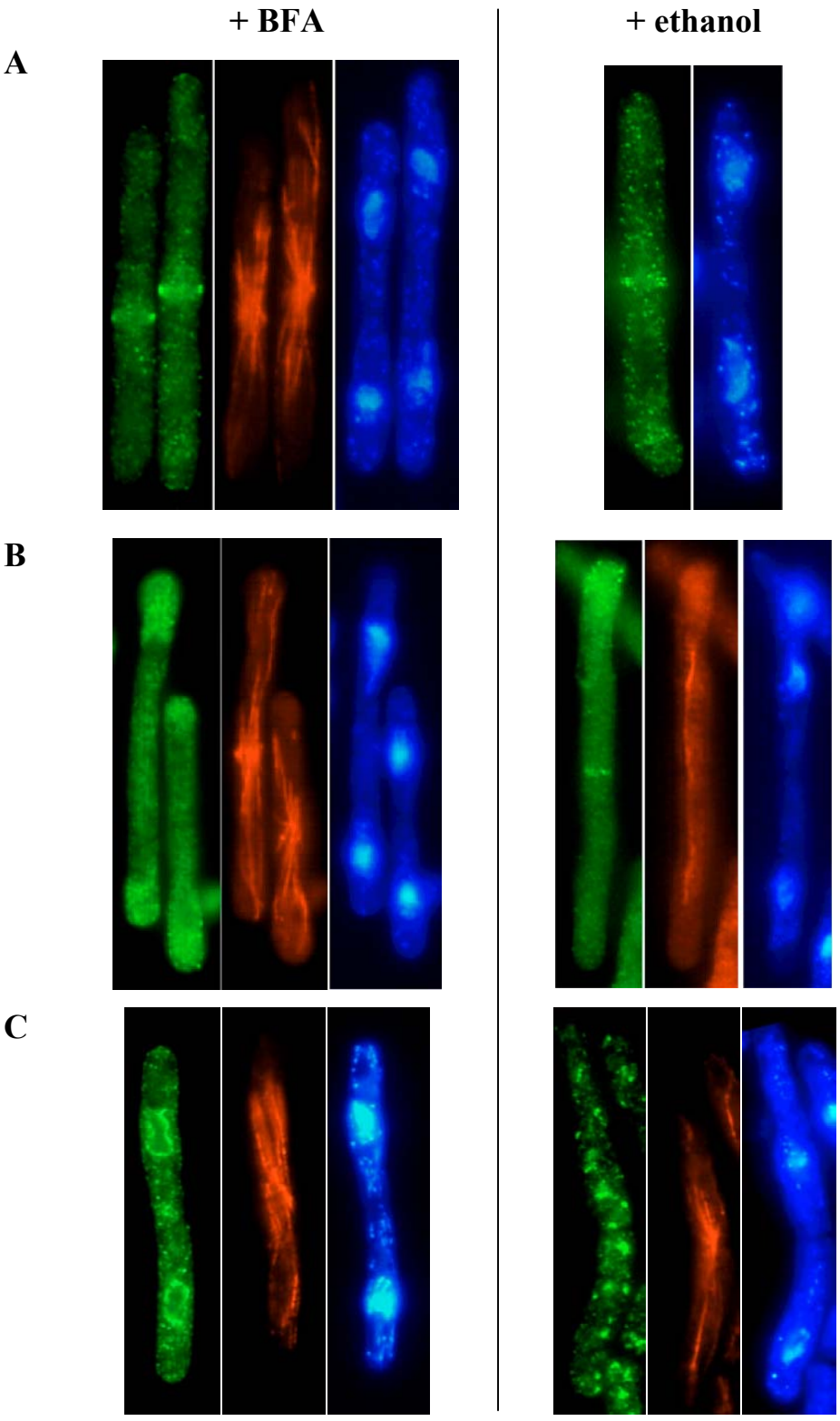


Figure 3.2.8.2-2 Sec6p can localize to the division site in *sec8*-1 cells. *sec8*-1 cells expressing Sec6-Myc were grown to log phase at 24°C, shifted to 36°C for 4 hrs and stained with DAPI and anti-Myc antibodies to visualize DNA and Sec6-Myc, respectively.

Figure 3.2.8.2-4 Sec10 localizes to the division site in *sec8*-1 cells. *sec8*-1 cells expressing Sec10-GFP were grown to log phase at 24°C, shifted to 36°C for 4 hrs and stained with DAPI and anti-GFP antibodies to visualize DNA and Sec6-GFP, respectively.

Figure 3.2.8.2-2

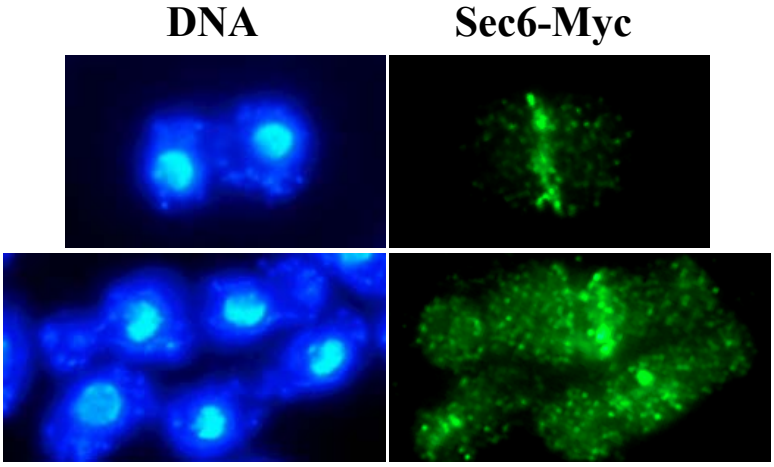


Figure 3.2.8.2-4

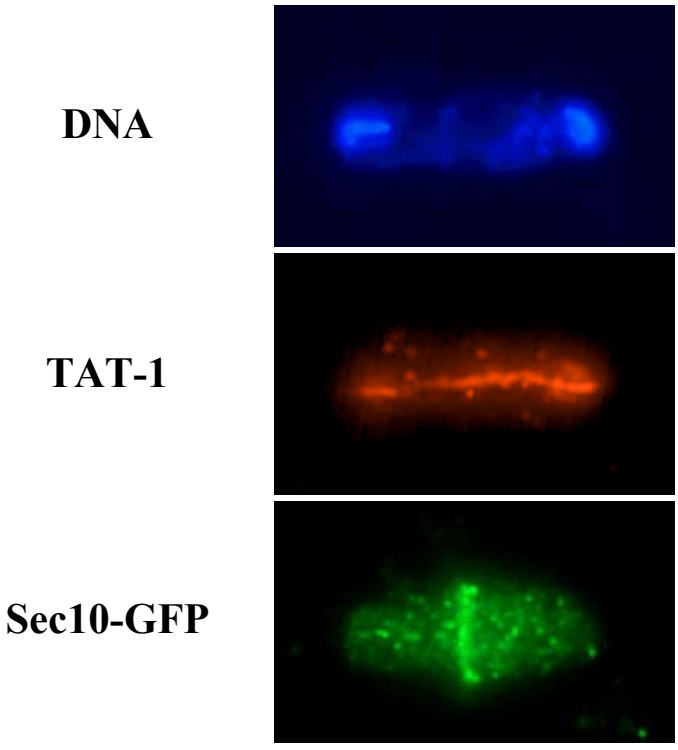
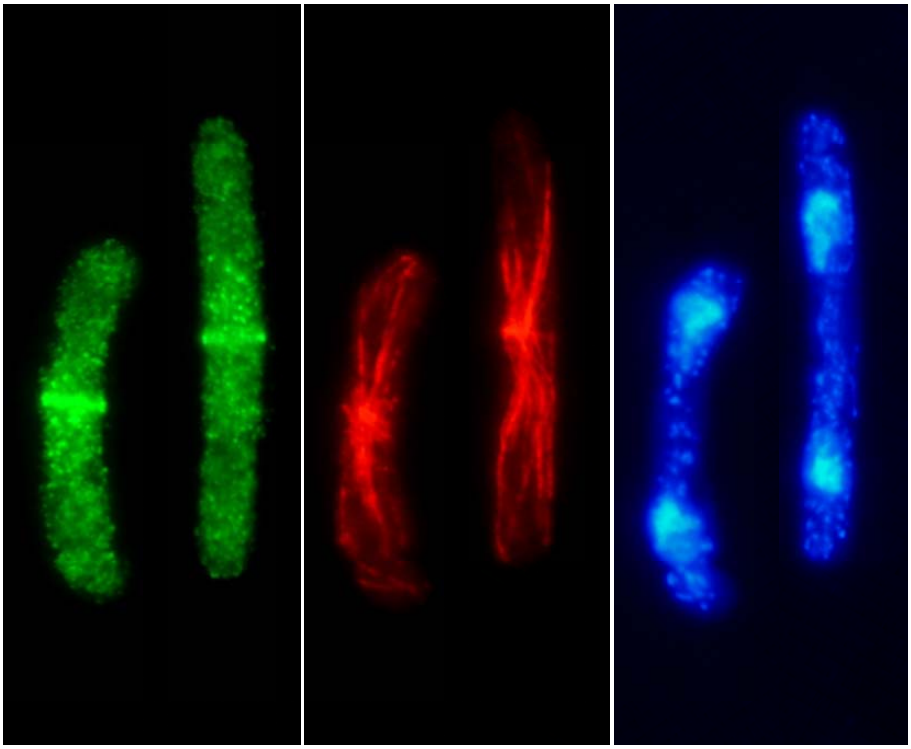


Figure 3.2.8.2-3 The localization of Sec10-GFP is independent of secretion. *cdc25*-22 cells expressing Sec10-GFP were treated with either BFA or ethanol upon released into mitosis. Samples were stained to visualize Sec10-GFP and microtubules.

Fig 3.2.8.2-3

+BFA



+ethanol

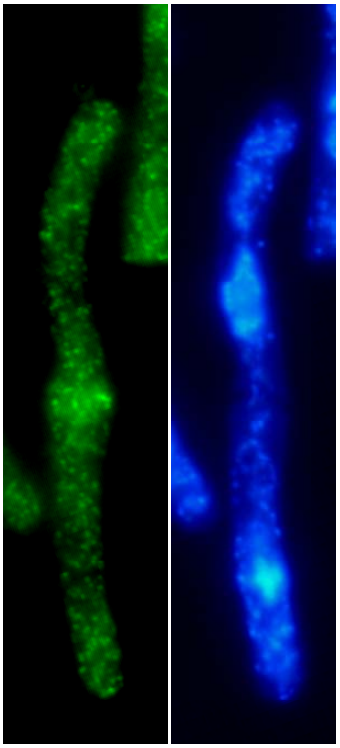
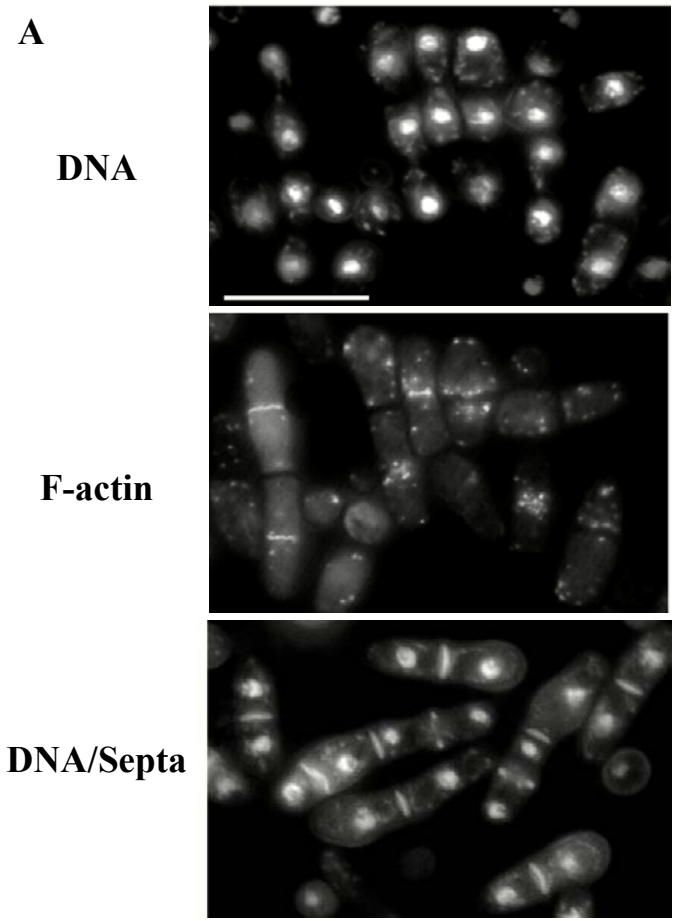


Figure 3.2.9.1 The phenotype of the exocyst null mutants. (A) A heterozygous diploid carrying *sec8*Δ marked with *ura4* gene were germinated in medium selecting for *sec8*Δ at 30°C. Cells were stained to visualize DNA, F-actin or co-stained for DNA/septum. (B) A heterozygous diploid carrying *sec6*Δ marked with *ura4* gene were germinated in medium selecting for *sec6*Δ at 30°C. Cells were stained to visualize DNA, F-actin and septum, respectively.

Fig 3.2.9.1



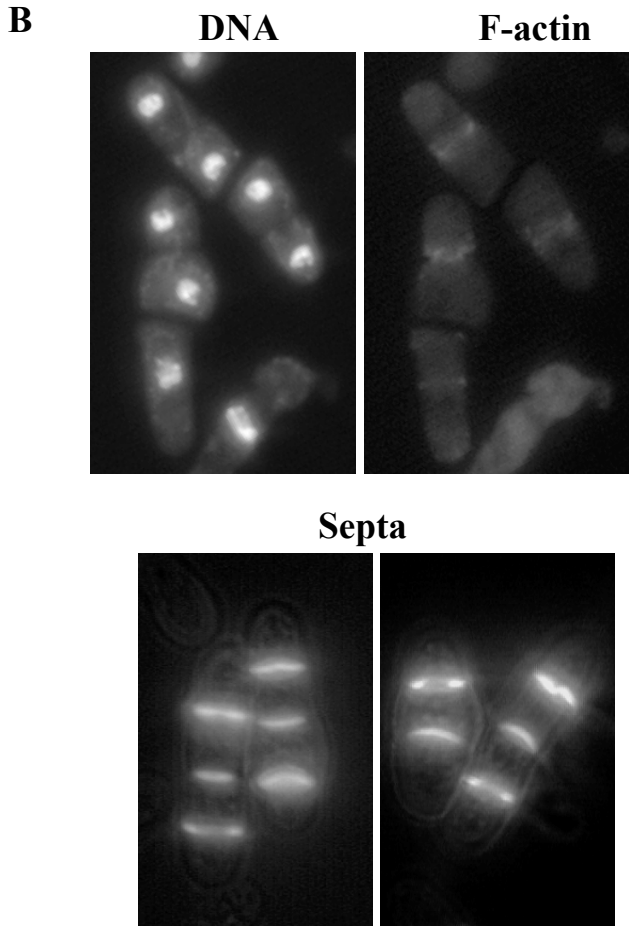


Figure 3.2.9.1 (C) A heterozygous diploid carrying *sec10*Δ marked with *ura4* gene were germinated in medium selecting for *sec10*Δ at 30°C. Cells were stained to visualize DNA, F-actin and septum, respectively. (D) Phenotype of *exo70Δ*. To compare the colony formation (upper panles), a wild-type strain and a haploid strain carrying *exo70Δ* marked with *ura4* gene were streaked out on plates and incubates at 24°C and 36°C, respectively. *exo70Δ* cells were grown to log phase at 24°C and shifted to 36°C for 6 hrs (lower panels). Cells from both temperatures were stained with DAPI/ aniline blue to visualize DNA/septum.

Figure 3.2.9.1

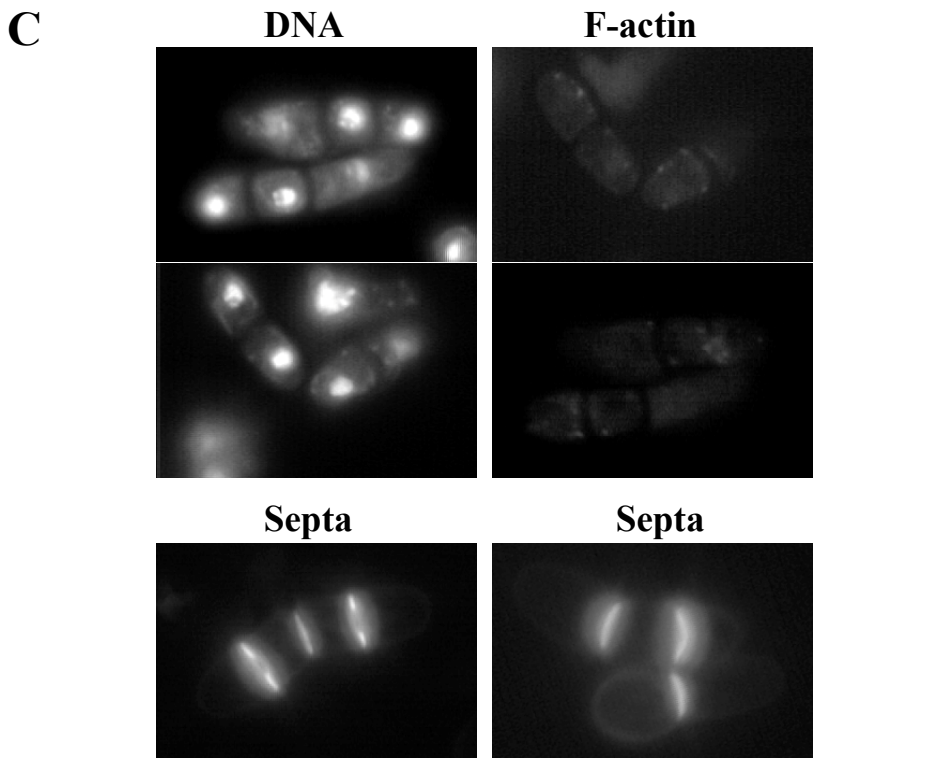
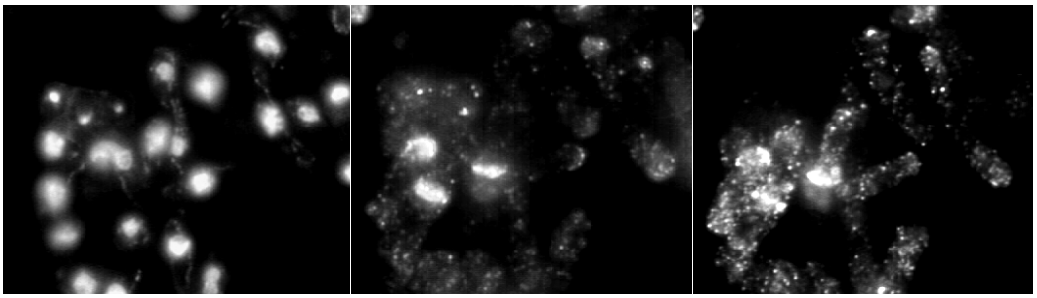

C
DNA
F-actin
Septa
Septa
D
Wild type
exo70Δ
Wild type
exo70Δ
24°C
36°C
DNA/Septum, 24°C
DNA/Septum, 36°C

Figure 3.2.9.2 The maternal Sec8p is not detected in germinating *sec8*Δ cells. A heterozygous diploid with one *sec8* locus tagged with Myc, and the other *sec8* locus replaced by the *ura4* gene was germinated to select for *sec8*Δ or Sec8-Myc, respectively. Cells were stained with antibodies against Myc and Mok1 to visualize Sec8-Myc and the α-glucan synthase Mok1p, respectively (Katayama et al, 1999).

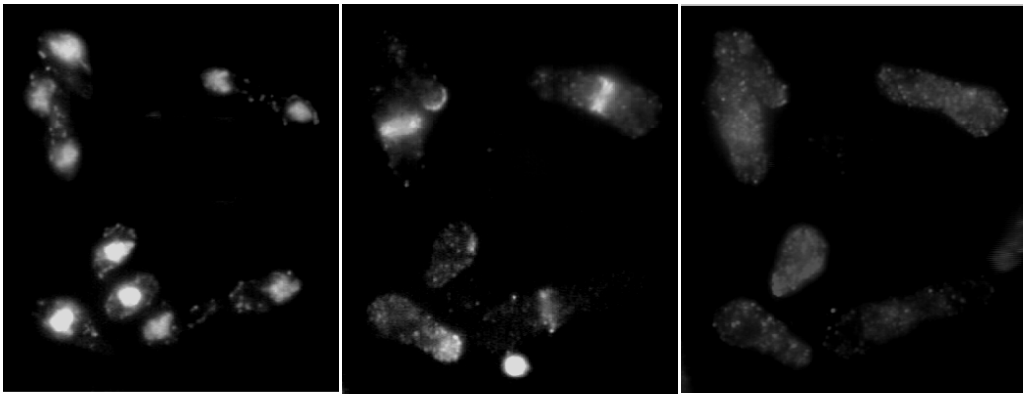
Fig 3.2.9.2

***sec8*-Myc**



DNA **Mok1p** **Sec8-Myc**

***sec8*-null**

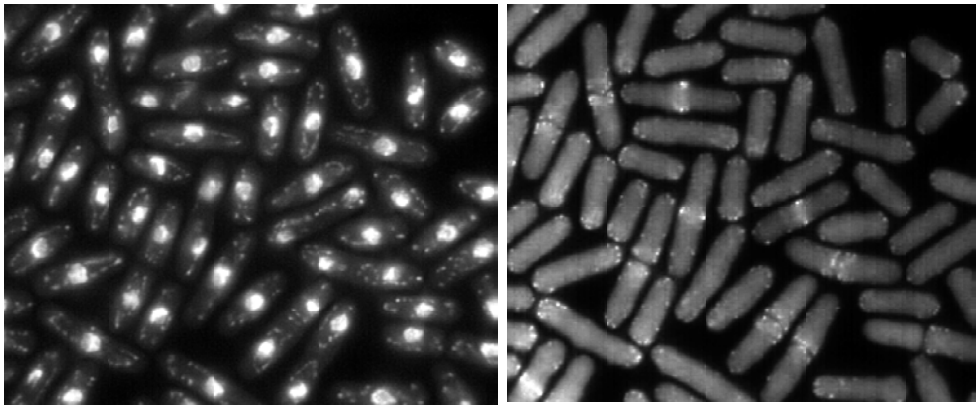


DNA **Mok1p** **Sec8-Myc**

Figure 3.2.10 Shutting off *sec8* expression causes defects in cell separation. *sec8* shut-off cells were grown in medium lacking thiamin (Sec8p expressed) or with thiamin (Sec8p repressed) at 30°C, and stained to visualize DNA/septum and F-actin.

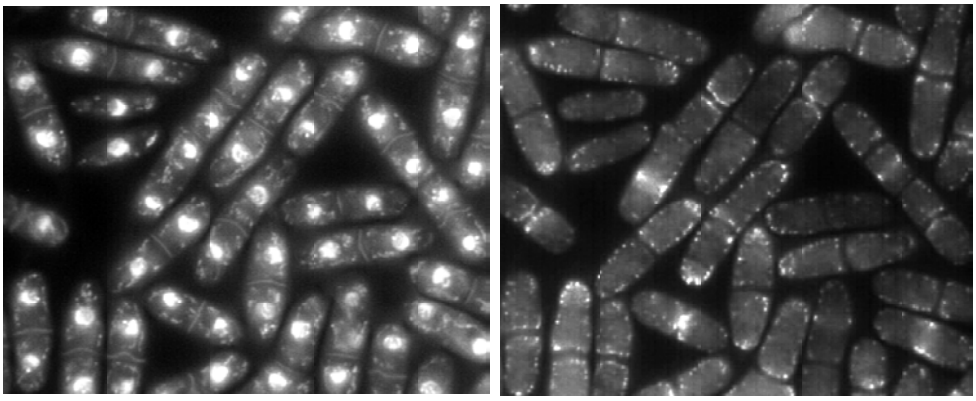
Fig 3.2.10

Sec8p expressed



DNA **F-actin**

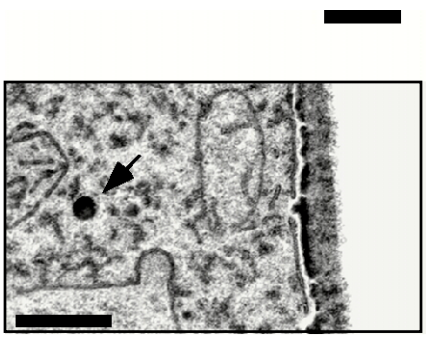
Sec8p repressed

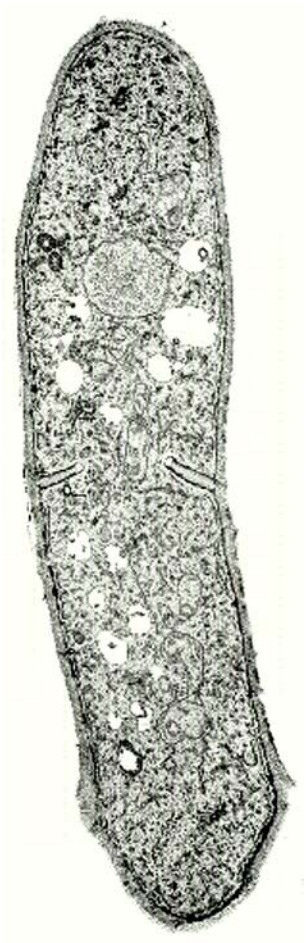


DNA/septa **F-actin**

Figure 3.2.11.1-1 Ultra-structural analysis of wild-type *S. pombe* cells. Wild-type cells were fixed in permanganate and subjected to electron microscopic analysis to visualize putative secretory vesicles. Cells in interphase, mitosis and cytokinesis are shown. The boxed region is enlarged on the side.

Fig 3.2.11.1-1





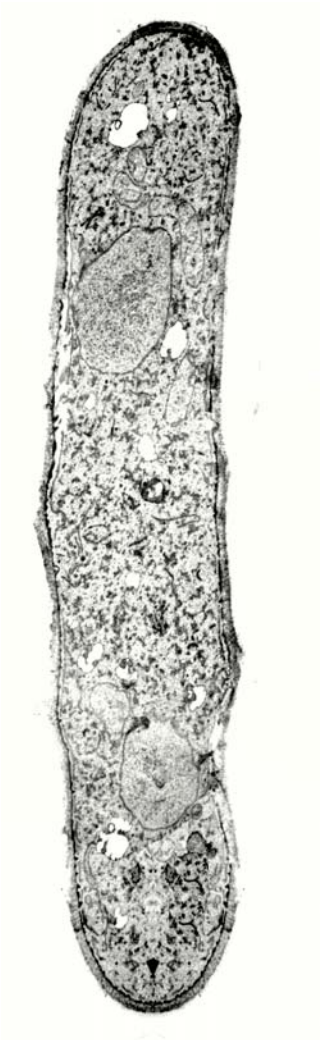
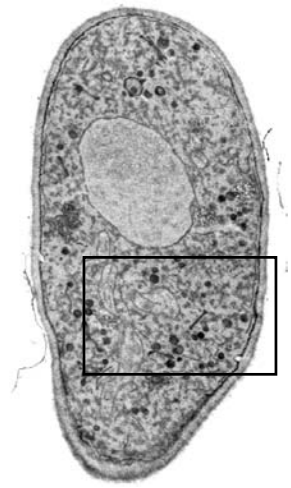
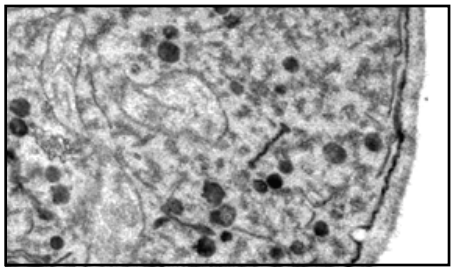
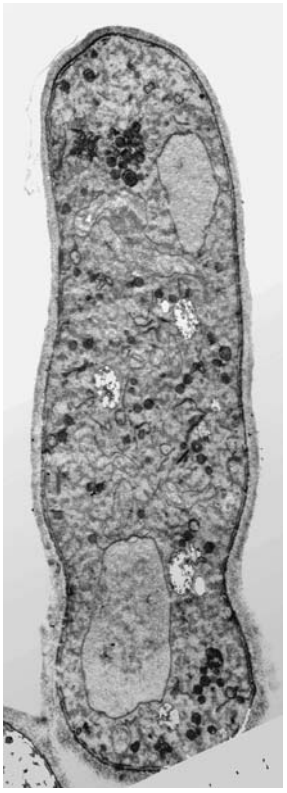


Figure 3.2.11.1-2 Ultra-structural analysis of *sec8*-1 cells. *sec8*-1 cells were grown at 24°C to exponential phase followed by a shift to 36°C for 4 hrs and were fixed and processed for electron microscopy. Cells at different cell cycle stages are shown. Putative secretory vesicles were stained as dark circles.

Fig 3.2.11.1-2







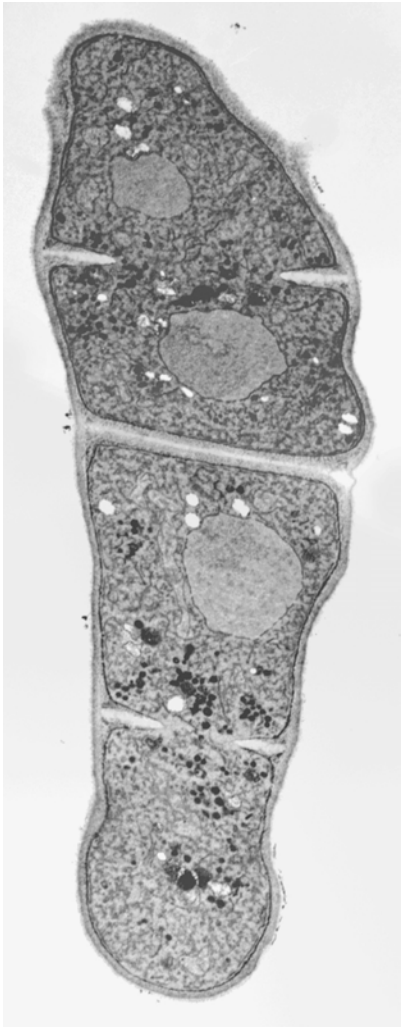
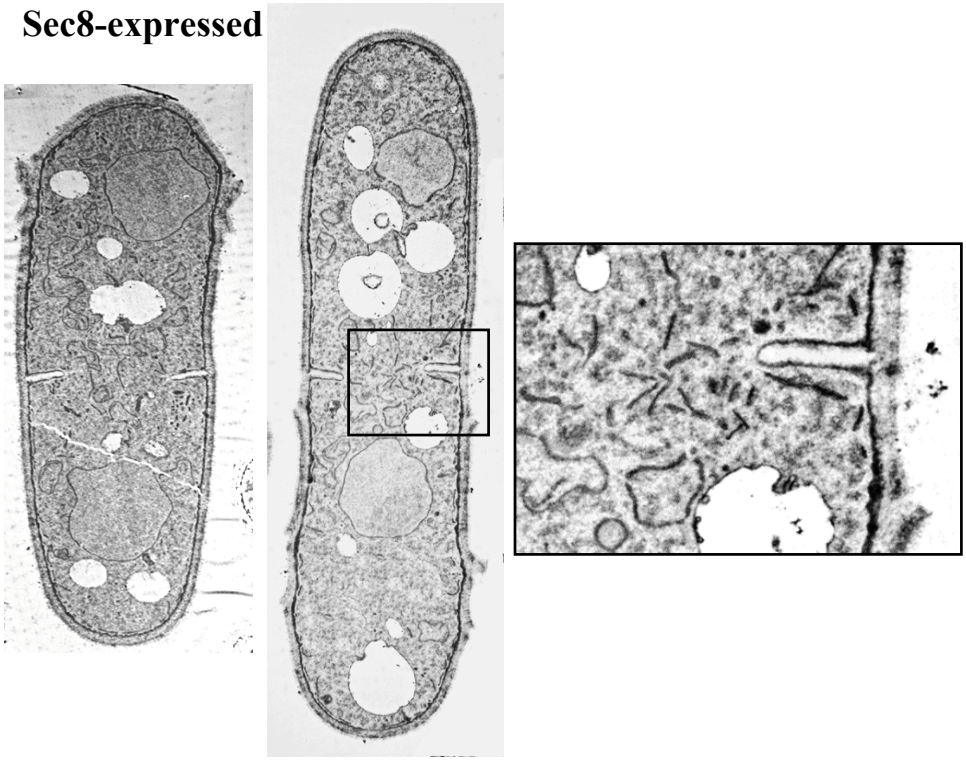


Figure 3.2.11.2 Electron microscopic analysis of *sec8* shut-off cells. *sec8* shut-off cells were grown in medium lacking thiamin (A) or in the presence of thiamin (B), fixed and processed for electron microscopy. Boxed regions are magnified on the side. Putative secretory vesicles appears as dark circles.

Fig 3.2.11.2

Sec8-expressed



Sec8-shut off

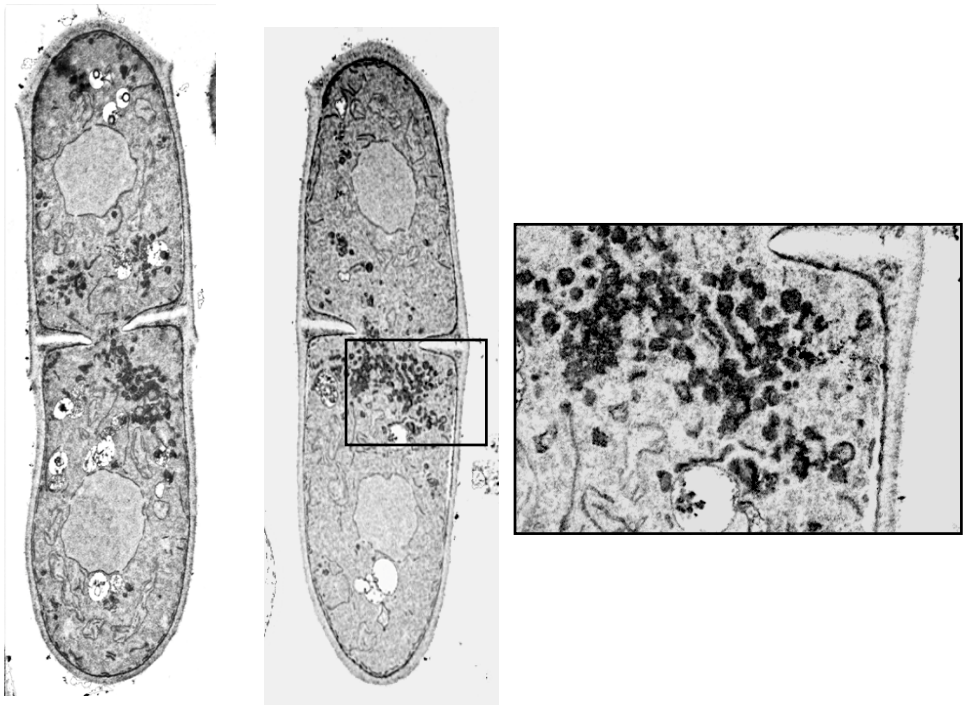


Figure 3.2.12 *sec8-1* displays lower activity of secreted acid phosphatase. Wild-type (squares) and *sec8-1* (triangles) cells were assayed for secreted acid phosphatase activity at 24°C (open) and 36°C (filled). *ypt2*Δ (circles) was included as a control that secrets less activity of acid phosphatase.

Fig 3.2.12

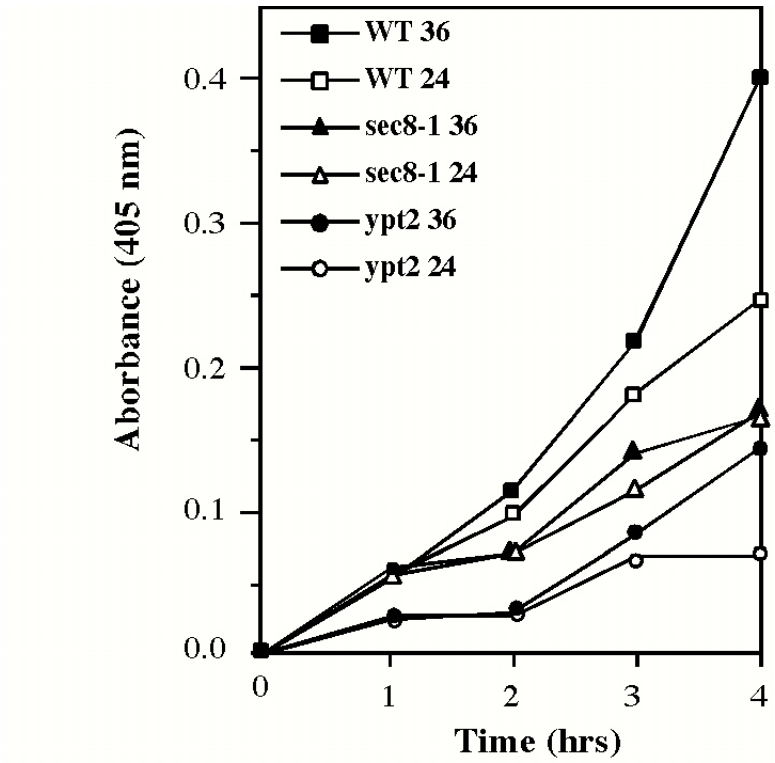
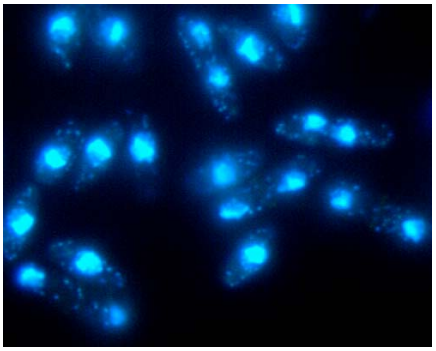

WT 36
WT 24
sec8-1 36
sec8-1 24
ypt2 36
ypt2 24
Aborbance (405 nm)
0.0
0.1
0.2
0.3
0.4
0
1
2
3
4
Time (hrs)

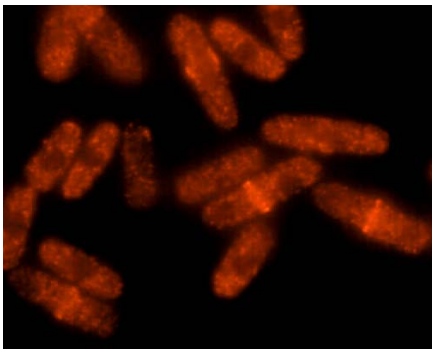
Figure 3.2.13 Co-localization of the exocyst with septins in *S. pombe*. Cells expressing both Sec10-Myc and Spn1-GFP were stained with antibodies against Myc and GFP to visualize Sec10p and Spn1p on the same cells.

Figure 3.2.13

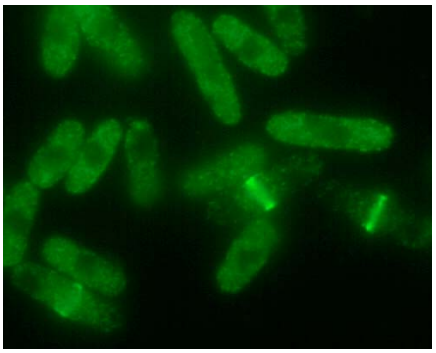
DNA



Sec10-Myc



Spn1-GFP



3.3 Discussion

3.3.1 An exocyst complex in the fission yeast *Schizosaccharomyces pombe*

Previous studies of cytokinesis in the fission yeast *Schizosaccharomyces pombe* have focused on actomyosin ring assembly, actin patch movement, signaling events that control septum delivery, and on the study of enzymes responsible for septum assembly (Simanis, 1995). However, little information was available on the regulation of cell separation. This study describes the isolation of *sec8*-1, a mutant that is defective in cell separation following assembly of the division septum. Molecular cloning established that Sec8p is a component of the exocyst protein complex with homologues in several other organisms including the prototypic Sec8p from the budding yeast *S. cerevisiae*. The exocyst is a multi-protein complex that has been identified in a number of organisms (Hsu et al., 1999). In budding yeast the exocyst consists of 7 core subunits; Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p and Exo84p (Guo et al., 1999; Potenza et al., 1992; TerBush et al., 1996). In addition, the budding yeast exocyst complex interacts with its targeting factor Sec3p and the rab-related GTPase Sec4p (Finger et al., 1998; Guo et al., 1999). Homologs of exocyst components have also been identified in other organisms including in mammalian cells (Kee et al., 1997). The exocyst proteins appear to be important for transport between the Golgi apparatus and the plasma membrane and have been implicated in targeting and fusion of Golgi derived vesicles with the plasma membrane (Bowser and Novick, 1991; Hsu et al., 1999; Potenza et al., 1992; Roth et al., 1998).

Using sequences of the budding yeast exocyst proteins, proteins related to Sec6p, Sec10p, Sec15p and Exo70 in *S. pombe* were identified. These four proteins are approximately 20% identical in protein sequence with the budding yeast, plant and rat

counterparts. Using biochemical methods it was shown that Sec6p, Sec8p, Sec10p and Exo70p interact physically. Therefore, an exocyst-like complex is present in *S. pombe*. However, the *S. pombe* exocyst complex appears to lack proteins related to the budding yeast Sec5p and Exo84p. It will be interesting to test if proteins structurally related to the budding yeast Sec5p and Exo84p associate with the *S. pombe* exocyst complex. The fact that *sec8*-1 mutants accumulate ~100 nm vesicles at the restrictive temperature indicates that the exocyst complex in *S. pombe*, as in budding yeast and in mammalian cells, is important for exocytic events. The accumulation of ~100 nm vesicles in interphase as well as mitotic cells suggests that the exocyst might participate in exocytic events in all phases of the cell cycle.

3.3.2 The *S. pombe* exocyst localizes to regions of active secretion

I show that the fission yeast exocyst proteins localize to both cell tips as well as the site of cell division. In early mitosis, the exocyst colocalizes with the actomyosin ring, and later splits into two ring-like structures upon constriction of the actomyosin ring. It was shown that the localization of the exocyst complex to the division site is dependent on an intact F-actin cytoskeleton and on the molecules that are important for actomyosin ring assembly. Thus, the actomyosin ring might serve as a spatial landmark for targeting of the exocyst complex. It is also possible that the exocyst complex might be transported to the division site along the F-actin cables (Balasubramanian et al., 1996; Marks and Hyams, 1985; Pelham and Chang, 2001) that are attached to the actomyosin ring. The function of Cdc15p, an SH3 domain containing protein is also essential for assembly of the exocyst at the division site (Fankhauser et al., 1995). It is interesting to note that Cdc15p is also related to proteins of the PACSIN family which are important for membrane transport events

(Lippincott and Li, 2000). It is likely that Cdc15p might participate in membrane transport events pertaining to cytokinesis and might allow the targeting of proteins that specify exocytic events or allow other proteins that themselves utilize the exocytic pathway during cytokinesis. The exocyst colocalized with Myo2p, an actomyosin ring marker, in SIN mutants that are defective for septum formation, indicating that the exocyst localized early in mitosis in a manner independent of SIN signaling (data not shown).

The localization of the exocyst appears to be independent of secretion since disruption of the Golgi apparatus by treatment with brefeldin A does not impair the ability of the exocyst complex to localize to the actomyosin ring. The secretion independent localization of the *S. pombe* exocyst is different from the situation in *S. cerevisiae* where it has been shown that the localization of all members of the exocyst complex (with the exception of the targeting subunit, Sec3p) depends on the secretory pathway (Finger et al., 1998). Sec3p-like protein in *S. pombe* has not been identified. Thus, in the absence of a Sec3p-like protein, *S. pombe* may have an unidentified protein that may not have significant sequence similarity to *S. cerevisiae* Sec3p but might perform a function similar to *S.c* Sec3p. Further biochemical analysis to purify interacting proteins of the exocyst may help to identify such a protein in *S. pombe*. Alternatively, the other components might have evolved additional secretion-independent mechanisms to achieve their intracellular localization in *S. pombe*. Thus, the exocyst complex might localize to the division site in a secretion independent and F-actin dependent manner to direct exocytic events.

3.3.3 The *S. pombe* exocyst is critical for cell separation

Mutations in the *S. cerevisiae* exocyst members appear to block fusion of all post-Golgi vesicles with the plasma membrane. Consequently, these mutants are unable to expand the cell surface and perish due to failure of all exocytic events (Finger and Novick, 1997; Roth et al., 1998; TerBush et al., 1996; TerBush and Novick, 1995). In contrast, *S. pombe* exocyst mutants are capable of polarized growth, cell surface expansion, and division septum assembly. *S. pombe* exocyst mutants appear to be specifically defective in cleavage of the division septum and cell separation. Given the differences in the phenotypes of exocyst mutants in the two yeasts, the terminal phenotypes of the exocyst mutants were established using several approaches. The terminal phenotypes of a *sec8* temperature sensitive mutant, a *sec8* shut-off strain, as well as the terminal phenotypes of germinated *sec8*-null mutant spores were investigated. It was also established that the phenotype of *sec8*-null mutant spores is not likely to be influenced by maternal carry-over of Sec8p from the heterozygous diploid. In addition, null mutations in *sec6* and *sec10* also result in a phenotype identical to that observed in the *sec8* mutants. One possibility is that the exocyst is essential only for a subset of secretory events in *S. pombe*. This conclusion is similar to that obtained from studies in MDCK cells where it has been shown that the exocyst is only important for delivery of proteins to the basolateral membranes but not to the apical membranes (Grindstaff et al., 1998). Thus, the exocyst might be important for delivery of proteins important for septum cleavage, but not for proteins involved in cell elongation and division septum assembly.

Previously identified mutants, such as sep1, sep15, that are defective in cell separation are viable and able to grow to form ramified chains of cells, indicating that a block in cell separation does not result in a loss of viability. The fact that most of the subunits of the exocyst are essential for viability may be explain by the possibility that in the absence of exocyst function, cell wall degrading enzymes are mis-targeted to the cell surface, causing cells to lyse. Alternatively, the exocyst may have additional functions in cell elongation or septum assembly.

Alternatively, the exocyst might participate in all secretory events in wild-type *S. pombe* cells. In its absence, however, other pathways might substitute for the exocyst in some exocytic events. Previous studies have shown that additional mechanisms exist in budding yeast and mammalian cells for the delivery of proteins from the Golgi apparatus to the plasma membrane via early and recycling endosomes (Brachet et al., 1999; Luo and Chang, 2000; Mallard et al., 1998). Currently, it is unclear if transport from Golgi apparatus to the plasma membrane via endosomes requires exocyst function. In this model, the exocyst is rate-limiting for the delivery of proteins important for septum cleavage and is redundant with other mechanisms important for targeting proteins required for polarized growth and division septum assembly. A third possibility is that in all the mutants that were analyzed in this study, a low level of exocyst activity might persist that might be sufficient for cell elongation and division septum assembly, but not for cell separation. Another possibility is that the exocyst is required for assembly of division septum and that in its absence abnormal septa are assembled. Both the primary and secondary septum of exocyst mutants are much thicker than wild-type cells (Fig 3.2.11, Fig 3.2.11.1-2 and Fig 4.2.2) and in some cells septa (based on Calcofluor staining) are not formed completely in the

middle (Fig 3.2.9.1), indicating a possible defect in septum assembly in these mutants. Although both Cps1p (data not shown) and Mok1p (Fig 3.2.9.2) are localized in exocyst mutants, it is not known if the exocyst is required for the turn-over of Cps1p. If the exocyst mutants assemble abnormal division septum, it is likely that Eng1p is unable to cleave a septum with an abnormal structure, which may also contribute to the defects in cell separation in exocyst mutants. A further investigation of these possibilities will require a detailed analysis of the septum assembled in exocyst mutants. The isolation and characterization of a bank of temperature-sensitive mutant alleles of the various exocyst components, followed by detailed cell biological and biochemical characterization of these mutants using secretion assays will also help understand whether the exocyst is involved in septum formation.

Most recently, a glucanase Eng1p was found to be required for cell separation (Martin-Cuadrado et al., 2003). Interestingly, Eng1p carries a signal sequence, suggesting it may be transported through secretory pathway (Martin-Cuadrado et al., 2003). Thus, it will be of great interest to investigate whether the targeting of Eng1p to the division site during cytokinesis is mediated by the fission yeast exocyst proteins.

Chapter IV Characterization of Rho3p in *S. pombe*

4.1 Introduction

The Ras GTPase proteins including members of the Rab, Rho and Ral families, have attracted increasing attention recently for their role in the regulation of exocytosis (Novick and Guo, 2002). As described in chapter 1.4.4**,** Sec4p, a member of the Rab family, is the first GTPase found to interact with the exocyst. Rho family members are also found to interact with the exocyst in *S. cerevisiae* (Guo et al., 1999). Independent of its function in organizing actin cytoskeleton, Rho1p regulates membrane trafficking through a direct binding of Sec3p (Guo et al., 2001). Another Rho family protein Cdc42p also interacts with Sec3p and this interaction targets Sec3p to the emerging bud (Zhang et al., 2001). Rho1p and Cdc42p competes for binding of Sec3p *in vitro* and may interact with Sec3p at different stages of the cell cycle *in vivo* (Zhang et al., 2001). In *S. cerevisiae*, Rho3p regulates vesicle transport via an interaction with Exo70p and the interaction is dependent on Rho3p effector domain and requires the GTP-bound Rho3p, suggesting that Exo70p is the downstream effector of Rho3p to mediate exocytosis (Robinson et al., 1999). To date, there are no report linking a Rho family protein to the mammalian exocyst. However, a mammalian GTPase RalA binds an exocyst component Sec5p, to effect vesicle transport (Sugihara et al., 2002).

A temperature-sensitive mutant identified previously, *sec8*-1, is defective in cell separation and vesicle trafficking in *S. pombe*. The *rho3* gene was isolated as a multi-copy suppressor of the temperature-sensitive exocyst mutation *sec8*-1 in the process of cloning *sec8*. Sequencing of the plasmid insert revealed that it contained the *rho3* gene, encoding a rho GTPase protein.

This chapter described the characterization of a small ras GTPase protein in *S. pombe*, namely Rho3p. Construction and analysis of a *rho3* deletion mutant as well as a dominant-negative mutant established that Rho3p, like the exocyst proteins, is required for cell separation. Rho3p is also involved in a late stage of membrane traffic in *S. pombe*.

4.2 Results

4.2.1 Rho3p is a small ras superfamily GTPase

A sequence homology search using the *S. pombe* Rho3p as query revealed that it is most related to fungal *Eremothecium gossypii* Rho3p, with these two proteins sharing 66% identity and 77% similarity along the entire sequences. It is also closely related to *S. cerevisiae* Rho3p (64% identity and 73% similarity with their amino acid sequences) and other ras superfamily proteins from mammals. The alignment of the *S. pombe*, *E. gossypii* and *S. cerevisiae* Rho3p is shown in Fig 4.2.1.

4.2.2 Overexpression of Rho3p suppresses all deleterious phenotypes associated with *sec8*-1

To confirm the suppression of *sec8*-1 by overproduction of Rho3p, a plasmid pREP3-1 carrying the *rho3* gene was re-introduced into *sec8*-1, and the transformed strain was streaked to single colonies on minimal medium selecting for the plasmid. All strains were incubated at either 24°C or 36°C for 2-3 days to access colony formation. A control plasmid without any insert (pREP3-1) and a plasmid carrying a random insert (pREP3-1-*pot1*) were also introduced into *sec8*-1. As shown in Figure 4.2.2 A, *sec8*-1 cells that were transformed with *rho3*$^{+}$ grew well and formed healthy colonies at 36°C, whereas cells transformed with control plasmids were unable to do so. Plasmids from

this rescued strain were extracted again and the sequence of the insert was determined to be the *rho3* gene (data not shown). Thus, the *rho3*$^{+}$ gene is a multi-copy suppressor of the *sec8*-1 mutation.

Given that the growth phenotype of *sec8*-1 was rescued by overexpression of Rho3p, I ascertained whether the cell separation defect of *sec8*-1 mutant could be suppressed by overproduction of Rho3p. *sec8*-1 cells overproducing Rho3p or control plasmids were grown at 24°C followed by a shift to 36°C for 4 hrs. Cells were stained with DAPI and aniline blue to visualize DNA and septum. Whereas *sec8*-1 cells carrying the vector alone, (Fig 4.2.2 B, upper panel) were unable to separate the division septum following cytokinesis, *sec8*-1 cells overexpressing Rho3p were capable of separation following cytokinesis and grew normally (Fig 4.2.2 B, lower panel). These data indicate that the cell separation phenotype of *sec8*-1 is suppressed by overproduction of Rho3p.

I next examined whether the secretion defect associated with *sec8*-1 could also be suppressed by overexpression of Rho3p. As previously described (Wang et al., 2002). *sec8*-1 cells accumulate presumed secretory vesicles (~100 nm in diameter, intensely stained after permanganate fixation) in the cytoplasm due to a defect in post-Golgi vesicle trafficking. *sec8*-1 cells overexpressing Rho3p or vector alone were grown at 24°C, shifted to 36°C for 4 hrs and processed for ultra-structural analysis. As expected, control cells accumulated a large number of putative secretory vesicles in the cytoplasm, resembling that of *sec8*-1 alone at 36°C (Fig 4.2.2 C left panel and upper box c1). Interestingly, very few such secretory vesicles were detected in *sec8*-1 cells overexpressing Rho3p (Fig 4.2.2 C right panel and lower box c2), suggesting that

overproduction of Rho3p can also rescue the secretion defect of *sec8*-1. Thus, all deleterious phenotypes associated with *sec8*-1 that were examined were suppressed by overproduction of Rho3p.

4.2.3 *rho3* is unable to suppress *sec8* null mutant

The suppression of *sec8*-1 mutant by ectopic expression of Rho3p could be explained by two possibilities. One possibility was that increased levels of Rho3p might bypass the requirement for Sec8p function. In this case, high dosage expression of Rho3p would rescue a *sec8*-null mutation. Alternatively, high levels of Rho3p might stimulate residual activity of the mutant Sec8-1p. If so, high dosage expression of Rho3p would not rescue the *sec8Δ*. To distinguish between these possibilities, it was investigated whether expression of a high-copy number *rho3* gene was able to suppress a *sec8Δ* mutant. A heterozygous diploid carrying a *sec8* null mutation marked with *ura4*$^+$ was transformed with a plasmid carrying *rho3* marked with *leu1*$^+$, sporulated, and spores were germinated in minimal medium selecting for the *sec8Δ* and the plasmid carrying *rho3*. These germinated spores (Fig 4.2.3, left panel) were found to exhibit a cell separation defect, similar to that of the *sec8Δ* alone (Fig 4.2.3, right panel). Thus, overexpression of Rho3p was unable to rescue the loss of Sec8p, indicating that Rho3p suppresses *sec8*-1 mutant by stimulating residual function of the mutant Sec8-1p, rather than bypassing the requirement for Sec8p.

4.2.4 *rho3* null mutant is defective in cell separation

To examine if Rho3p had a role in cell separation, a *rho3* deletion mutant (*rho3Δ*) was constructed by replacing the *rho3* ORF with the *ura4* gene. *rho3Δ* formed colonies at

24°C, indicating that the *rho3* gene was not essential. Interestingly, *rho3Δ* cells exhibited a temperature sensitive phenotype. As shown in figure 4.2.4A, at the permissive temperature (24°C), *rho3Δ* cells grew well and formed healthy colonies, whereas at the restrictive temperature (36°C), the *rho3Δ* mutant grew very poorly and was unable to form colonies.

To examine the phenotype of *rho3Δ*, cells were grown at 24°C followed by a shift to 36°C for 4 hrs. Cells grown at both temperatures were fixed and stained with DAPI, phalloidin and Calcofluor to visualize DNA, F-actin and septum, respectively. At 24°C *rho3Δ* cells resembled wild-type cells, without any discernible phenotype (Fig 4.2.4B). However, following the shift to 36°C for 4hrs, *rho3Δ* cells were found to be defective in cell separation (Fig 4.2.4C). These cells were able to grow and assemble the division septa, but were defective in proper cleavage of septa. A majority of the mutant cells failed to separate and showed thickened septa despite normal mitosis and F-actin distribution. Upon prolonged incubation at 36°C (5 h to 16 h), *rho3Δ* cells displayed aberrant cell shapes and F-actin patches were delocalized all over the cell cortex (Nakano et al., 2002). This phenotype mimicked that of exocyst mutants, indicating that Rho3p is also required for cell separation, similar to the exocyst proteins.

4.2.5 *rho3Δ* interacts genetically with *sec8*-1 and *exo70Δ*

4.2.5.1 *rho3Δ* is synthetically lethal with *sec8*-1

To test genetic interactions between *rho3* and *sec8*, a *rho3Δ* strain was crossed to a *sec8*-1 mutant. Tetrads from the diploid were dissected on YES plates and incubated

at 24°C. Interestingly, the double mutant was inviable whereas both single mutants survived at 24°C, indicating that *rho3Δ* was synthetically lethal with *sec8*-1 (Fig 4.2.5.1). From this cross, there were 6 PD (parental ditype), 12 TT (tetrad type) and 3 NPD (non-parental ditype), in which the double mutant(s) died in each tetrad, suggesting a genetic interaction between Rho3p and Sec8p. This genetic interaction together with the high-copy suppression clearly establishes that Rho3p functions in coordination with the exocyst complex. However, no physical interaction was able to be biochemically reconstituted, possibly due to its relatively transient nature.

4.2.5.2 *rho3Δ* interacts genetically with *exo70Δ*

To explore whether *rho3* interacted with other exocyst components, I examined genetic interactions between *rho3* and another exocyst mutant *exo70Δ,* which exhibited a temperature-sensitive lethal phenotype and had a cell separation defect at 36°C. It was tested whether overexpression of Rho3p could also suppress *exo70Δ*. *exo70Δ* cells were transformed with plasmids pREP3-1 carrying *rho3* gene or pREP3-1 alone and plated at 36°C. *exo70Δ* cells overexpressing Rho3p were able to grow well at 36°C, whereas *exo70Δ* cells carrying the control plasmid did not grow under the same conditions. Thus, overproduction of Rho3p was able to suppress the growth defect of *exo70Δ* (Fig 4.2.5.2A). *exo70Δ* cells overexpressing Rho3p or the control plasmid were grown at 24ºC, shifted to 36ºC for 4hrs and stained with DAPI and aniline blue to visualize DNA and septum, respectively. In *exo70Δ* cells overexpressing Rho3p, the primary septum of most of cells are partially dissolved, with two compartments are still connected, suggesting the cell separation phenotype of *exo70Δ* was only partially suppressed by overexpression of Rho3p (data not shown).

Given that both Rho3p and Exo70p are non-essential for cell viability, I wanted to ascertain whether the simultaneous depletion of both proteins would result in more severe phenotype than either single mutant. The double mutant *rho3Δ exo70Δ* failed to grow at 32ºC, which was permissive for either of the single mutants (Fig 4.2.5.2B) further confirming this genetic interaction. While *rho3Δ exo70Δ* cells showed a mild cell separation defect at 24ºC (Fig 4.2.5.2C, left panel), this phenotype was exacerbated at 32ºC (Fig 4.2.5.2C, right panel) with most cells arresting with multiple septa, similar to *sec8*-1 mutant phenotype, confirming a genetic interaction between Rho3p and Exo70p. Thus, *S. pombe rho3* shows strong genetic interactions with all exocyst components for which conditionally lethal mutants are currently available.

4.2.6 Phenotypes of dominant *rho3* mutations

Rho3p is a member of a family of small GTPases that function as molecular switches. Given that activating and inactivating mutations have been extensively characterized in several ras-related GTPases, to study the function of Rho3p, dominant-active and dominant-inactive forms of Rho3p were characterized by constructing mutations in *rho3* (Fig 4.2.6) that are analogous to ras oncogene.

4.2.6.1 The dominant-active form of Rho3p

To analyze the dominant – active phenotypes of *rho3* alleles, a mutation in *rho3* that predicts a constitutively active form (Rho3G25V) was constructed by oligo-directed mutagenesis. This mutant form of Rho3p was expressed under a high-strength *nmt1* promoter, and cells were grown at 30°C for 24 hrs, fixed and stained with DAPI and Calcofluor to visualize DNA and septum, respectively. Cells overexpressing

Rho3G25V (Fig 4.2.6.1) displayed defects in morphology and cell wall integrity. Approximately 10% of cells had morphological defects, with one tip bulged. Approximately 30% cells had excessive cell wall materials in the cell, mostly seen at cell tip(s) and medial region. In addition, the position of the division septum was altered in some cells (arrow). This indicated that Rho3p might be required for cell polarity and cell wall integrity.

4.2.6.2 The dominant-inactive form of Rho3p

Similarly, a dominant – inactive form of Rho3p (Rho3T30N) was generated and expressed under the control of the thiamin repressible promoter nmt1 (high strength). Cells overproducing Rho3T30N (Fig 4.2.6.2) were arrested with multiple septa without proper cleavage. Mitosis and septation continued normally, indicating that they were defective in cell separation, as observed with the *rho3Δ* mutant. This result further confirmed that Rho3p is involved in cell separation.

4.2.7 The localization of exocyst proteins is independent of Rho3p

A possible role for Rho3p in cell separation could be regulation of the localization of the exocyst at the division site. Thus, the localization of exocyst components Sec8p and Sec6p was examined in a *rho3Δ* strain. *rho3Δ* cells expressing Sec8p-GFP were grown at both 24°C (Fig 4.2.7, left panel) and 36°C (Fig 4.2.7, right panel) and examined for Sec8p-GFP epifluorescence. At both conditions, Sec8p-GFP was clearly localized to the division site and growing tip(s) in a *rho3Δ* background, suggesting that the localization of Sec8p was independent of Rho3p. Similarly, the localization of Sec6p was examined in a *rho3Δ* strain at 36°C and it also seemed to be unaffected in a *rho3Δ* mutant (data not shown). Based on these observations, it was proposed that

regulation of localization of the exocyst complex to the division site is not a likely function of Rho3p.

4.2.8 Localization of Rho3p

4.2.8.1 GFP-Rho3p expressed from native promoter did not show localization

To investigate the intracellular localization of Rho3p, the carboxyl-terminal of Rho3p was fused to *GFP* and expressed under its native promoter at its chromosomal locus. However, no discernible GFP signal could be observed in these cells (data not shown), presumably due to the low expression levels.

4.2.8.2 GFP-Rho3p localizes to the division site

I then fused the *rho3* gene at the amino terminus with *GFP* and expressed it from the *nmt1* promoter of pREP1 plasmid (Maundrell, 1990) and introduced this plasmid into wild-type cells as well as a *rho3*-deletion mutant strain. This plasmid expressing GFP-Rho3p under repressing conditions was able to rescue *rho3Δ* at 36°C (Fig 4.2.8.2A), indicating that this fusion protein was functional. Under conditions of *nmt1* promoter repression, the fusion protein was localized to the plasma membrane in interphase. During cell division, GFP-Rho3p was localized to the division site (Fig 4.2.8.2B) similar to exocyst components. This suggests that *rho3* is a plasma membrane bound protein that localizes to the division site during cytokinesis.

4.2.8.3 Rho3p localization partially requires functional exocyst

To investigate whether the localization of Rho3p required the function of the exocyst, the localization of Rho3p was examined in an exocyst mutant. *sec8*-1 cells were transformed with a plasmid expressing GFP-Rho3p and examined for GFP-Rho3p

epifluorescence at both 24°C and 36°C. Whereas GFP-Rho3p alone localized in wild-type cells (Fig 4.2.8.3A, upper panel), its localization was partially compromised in most *sec8*-1 cells at 36°C in that a significant proportion of the protein was detected in intracellular structures (Fig 4.2.8.3B, lower panel). Even at 24°C, Rho3p localization was compromised in a significant portion of *sec8*-1 cells (data not shown). These data indicate that efficient Rho3p localization to the plasma membrane depends on functional Sec8p, but that Rho3p localization is not solely dependent on exocyst function.

4.2.9 Rho3p is involved in vesicle transport

4.2.9.1 *rho3Δ* cells accumulate a large number of secretory vesicles

Given that *rho3* was isolated as a high-copy suppressor of a mutant defective in vesicle transport, I investigated whether Rho3p was also involved in vesicle transport. If Rho3p acts in a late stage in the secretory pathway, *rho3Δ* should be defective in targeting or fusing of secretory vesicles from the Golgi apparatus to the plasma membrane and would accumulate secretory vesicles in the cytoplasm. *rho3Δ* cells were grown either at 24°C or 36°C were fixed with permanganate and subjected to electron-microscopy analysis. In *rho3Δ* cells grown at 24°C, secretory vesicles were rarely observed (Fig 4.2.9.1A), similar to the observation in wild type cells (Wang et al., 2002). Interestingly, in *rho3Δ* cells growing at 36°C, a large number of putative secretory vesicles (~100 nm) accumulated in the cytoplasm in both uninucleate and binucleate interphase cells, suggesting that Rho3p might play a role in trafficking of secretory vesicles in all stages of the cell cycle (Fig 4.2.9.1B).

4.2.9.2 *rho3Δ* cells secrete diminished amounts of acid phosphatase at 36°C

To ascertain the role of Rho3p in exocytosis using a different approach, the transport of acid phosphatase, a protein that transits through the secretory pathway, was monitored in *rho3Δ* cells. The activity of acid phosphatase was assayed in both *rho3Δ* cells as well as wild-type cells at 24°C or 36°C. At 24°C, *rho3Δ* cells secreted comparable amount of acid phosphatase as that observed in wild-type cells. However, at 36°C, *rho3Δ* was found to secrete much less amount of acid phosphatase at 36°C compared to that observed in wild-type cells (Fig 4.2.9.2). These results suggest that Rho3p is very likely to be involved in post-Golgi vesicle trafficking, possibly by coordinating with the exocyst complex.

Figure 4.2.1 The alignment of *S. pombe* Rho3p with its related proteins from *E. gossypii* and *S. cerevisiae*. Identical amino acids are shaded in black and conservative substitutions are shaded in grey.

Fig 4.2.1

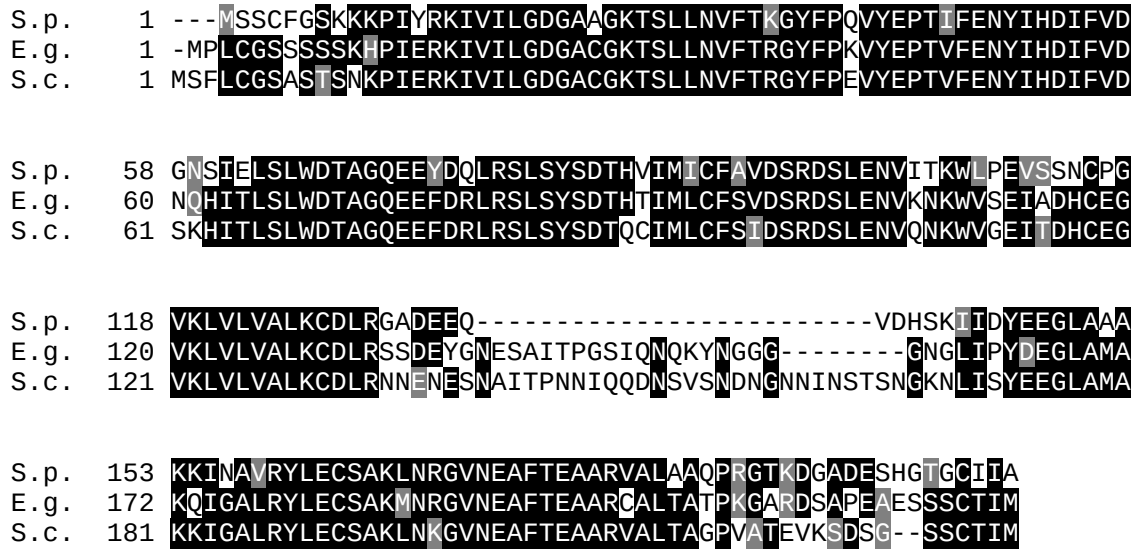
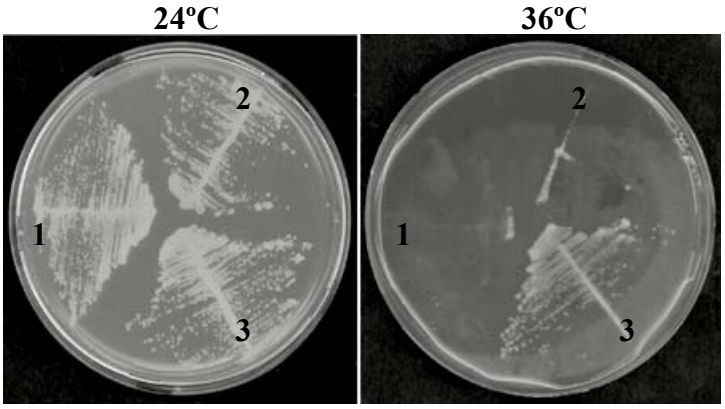

S.p. 1 ---MSSCFGSKKKPIYRKIVILGDGAAGKTSLLNVFTKGYFPQVYEPTIFENYIHDIFVD
E.g. 1 -MPLCGSSSSSKHPIERKIVILGDGACGKTSLLNVFTRGYFPKVYEPTVFENYIHDIFVD
S.c. 1 MSFLCGSASTSNKPIERKIVILGDGACGKTSLLNVFTRGYFPEVYEPTVFENYIHDIFVD
S.p. 58 GNSIELSLWDTAGQEEYDQLRSLSYSDTHVIMICFAVDSRDSLENVITKWLPEVSSNCPG
E.g. 60 NQHITLSLWDTAGQEEFDRLRSLSYSDTHTIMLCFSVDSRDSLENVKNKWVSEIADHCEG
S.c. 61 SKHITLSLWDTAGQEEFDRLRSLSYSDTQCIMLCFSIDSRDSLENVQNKWVGEITDHCEG
S.p. 118 VKLVLVALKCDLRGADEEQ-------------------------VDHSKIIDYEEGLAAA
E.g. 120 VKLVLVALKCDLRSSDEYGNESAITPGSIQNQKYNGGG--------GNGLIPYDEGLAMA
S.c. 121 VKLVLVALKCDLRNNENESNAITPNNIQQDNSVSNDNGNNINSTSNGKNLISYEEGLAMA
S.p. 153 KKINAVRYLECSAKLNRGVNEAFTEAARVALAAQPRGTKDGADESHGTGCIIA
E.g. 172 KQIGALRYLECSAKMNRGVNEAFTEAARCALTATPKGARDSAPEAESSSCTIM
S.c. 181 KKIGALRYLECSAKLNKGVNEAFTEAARVALTAGPVATEVKSDSG--SSCTIM

Figure 4.2.2 Overexpression of Rho3p suppresses all deleterious phenotypes associated with *sec8*-1. (A) *rho3* is a multi-copy suppressor of *sec8*-1 in *S. pombe*. A *sec8*-1 strain transformed with a blank control plasmid pREP3-1 (1), a random insert control plasmid pREP3-1-*pot1* (2) and *rho3* containing plasmid pREP3-1-*rho3* (3) were grown on plates at 24°C (left panel) for 4 days and 36°C (right panel) for 2 days, respectively. (B) Cell separation phenotype of *sec8*-1 is suppressed upon overproduction of Rho3p. *sec8*-1 cells carrying pREP3-1 (upper panel) or pREP3-1-*rho*3 (lower panel) were grown at 36°C and stained with DAPI / aniline blue to visualize DNA and septum. Bar, 10 μm.

Fig 4.2.2

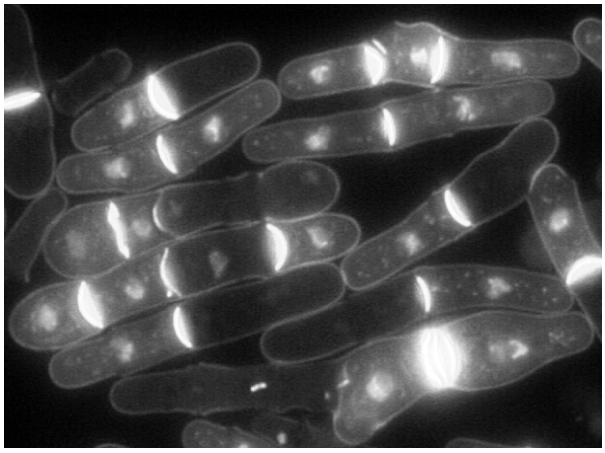
A



1. pREP3-1
2. pREP3-1-*pot1*
3. pREP3-1-*rho3*

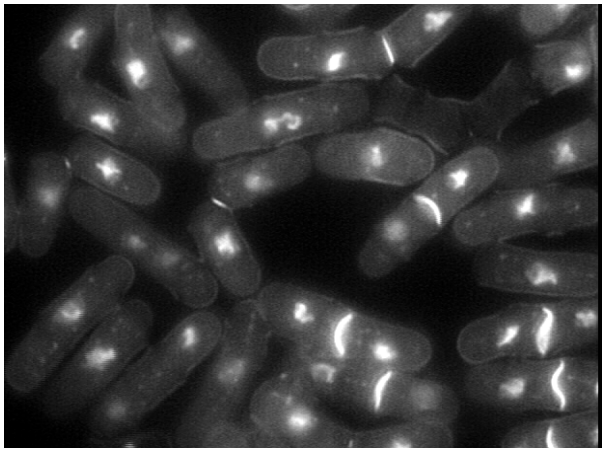
B

***sec8*-1+pREP1**



DNA/Septum

sec8*-1+pREP1-*rho3



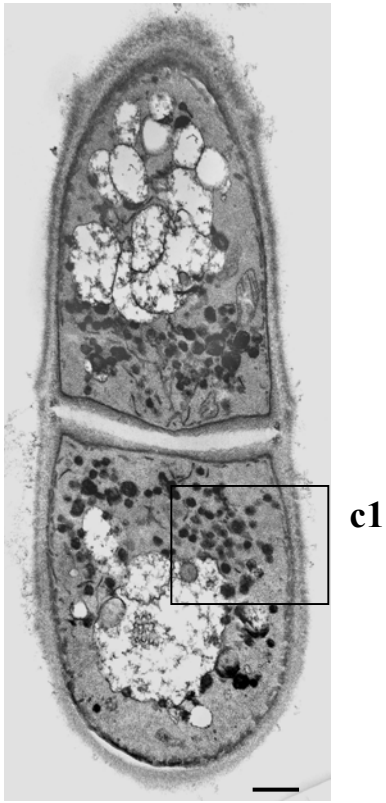
DNA/Septum

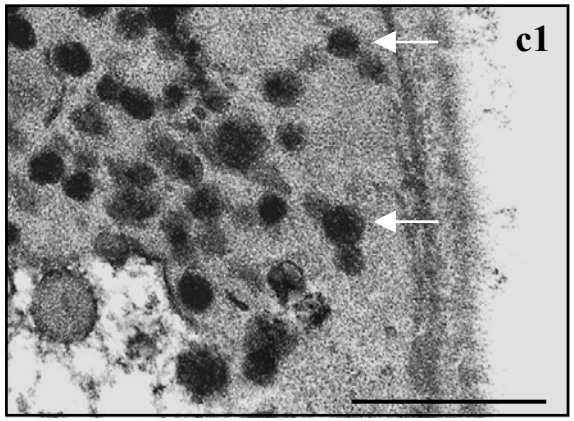
Figure 4.2.2 (C) *sec8*-1 cells carrying pREP3-1 (left panel) or pREP3-1-*rho*3 (right panel) were grown at 36°C, fixed and processed for electron microscopy. c1 and c2 are magnified regions. White arrows, putative secretory vesicles. Bar, 500μm.

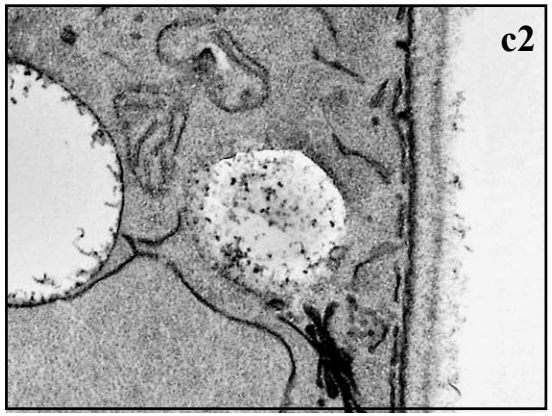
Fig 4.2.2

C

***sec8*-1+pREP1**







sec8*-1+pREP1-*rho3

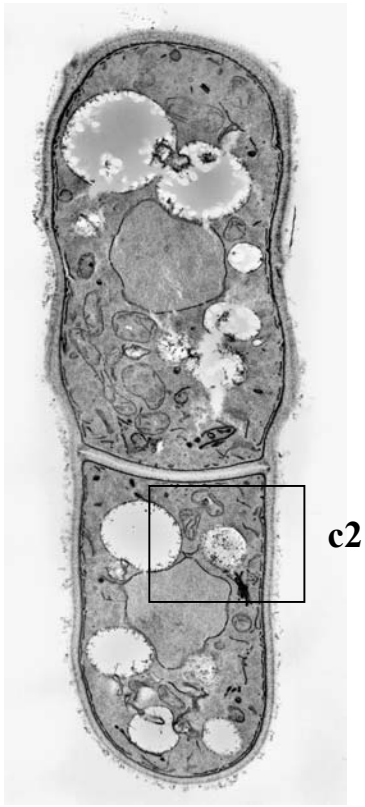


Figure 4.2.3 Overexpression of Rho3p is unable to suppress a *sec8Δ* strain. A diploid strain carrying *sec8Δ* mutation and a pREP3-1-*rho3* plasmid was sporulated and germinated in minimal medium lacking uracil and leucine to select for *sec8Δ* and pREP3-1-*rho3* plasmid at 30°C for 24 hrs. Cells were then stained with DAPI to visualize DNA. DAPI-stained *sec8Δ* are shown for comparison.

Fig 4.2.3

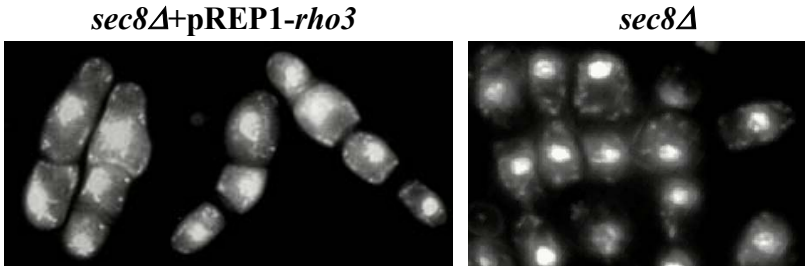
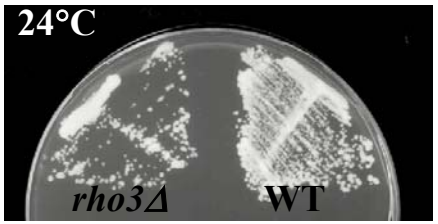
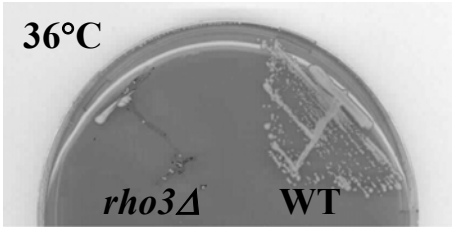


Figure 4.2.4 A *rho3Δ* mutant is defective in cell separation at the restrictive temperature. (A) To compare relative growth rates, *rho3Δ* (left) and wild-type (right side) cells were streaked to single colonies on plates and incubated at 24°C (upper panel) and 36°C (lower panel). (B-C) Phenotypes of *rho3Δ* cells. *rho3Δ* cells were grown at either 24°C (B) or shifted to 36°C for 4 hrs (C), fixed and stained with DAPI, Rhodamine-conjugated Phalloidin and Calcofluor to visualize DNA, F-actin and cell wall, respectively.

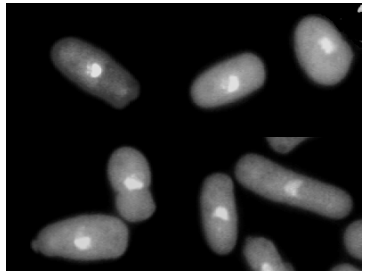
Fig 4.2.4

A

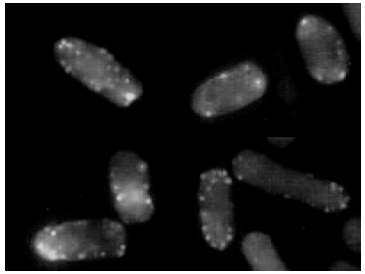




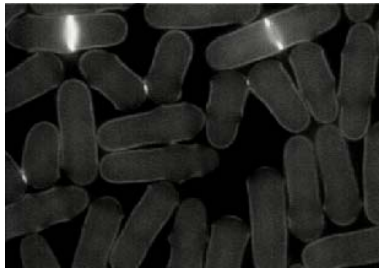
B



DNA



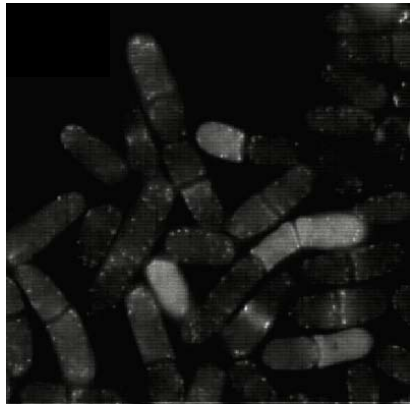
F-actin



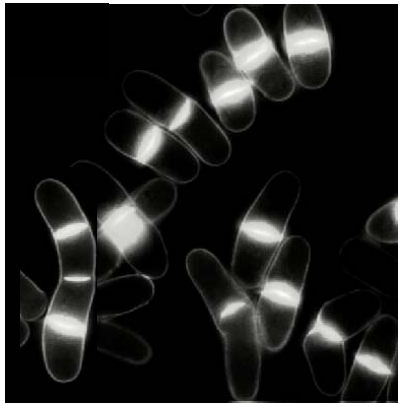
Septum

C

DNA



F-actin



Septum

Figure 4.2.5.1 *sec8*-1 is synthetically lethal with *rho3* deletion mutant. Asci from a cross of a *sec8*-1 mutant strain with a *rho3* deletion mutant strain were dissected on YES and grown at 24°C. One non-parental ditype (NPD) is shown. Double mutants were unable to form colonies at 24°C.

Fig 4.2.5.1

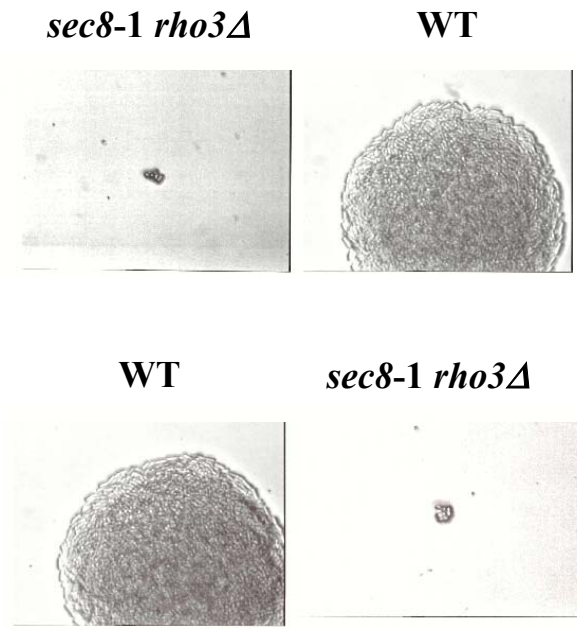
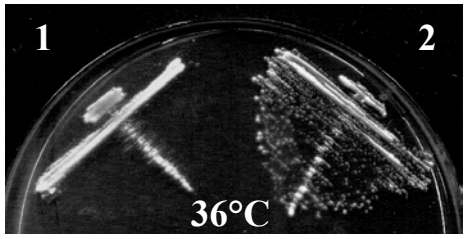


Figure 4.2.5.2 *sec8* interacts genetically with *exo70*. (A) Overproduction of Rho3p suppresses growth defects of *exo70Δ*. *exo70Δ* cells transformed with pREP3-1 (left side) or pREP3-1-*rho3* (right side) were grown on plates and were incubated at 24°C or 36°C. (B) *exo70* shows a negative genetic interaction with *rho3*. Strains *exo70Δ* (1), *rho3Δ* (2) and *exo70Δ rho3Δ* (3) were grown either at 24°C (left panel) or 36°C (right panel) for 3 days. (C) *exo70Δ rho3Δ* double mutant shows more severe defects in cell separation than each single null mutants. *exo70Δ rho3Δ* cells were grown at 24°C (left panel) or shifted to 32°C for 4 hrs (right panel), fixed and stained with DAPI / aniline blue to visualize DNA and septum.

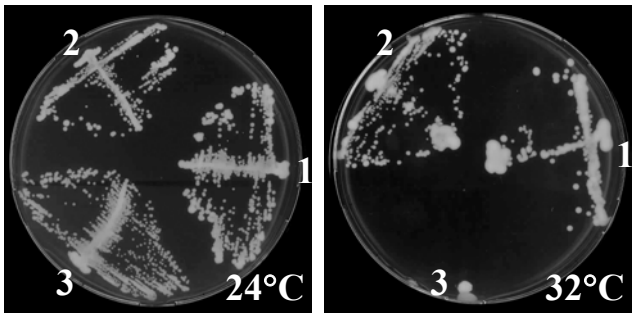
Fig 4.2.5.2

A



1. *exo70Δ*+pREP3-1
2. *exo70Δ*+pREP3-1-*rho3*

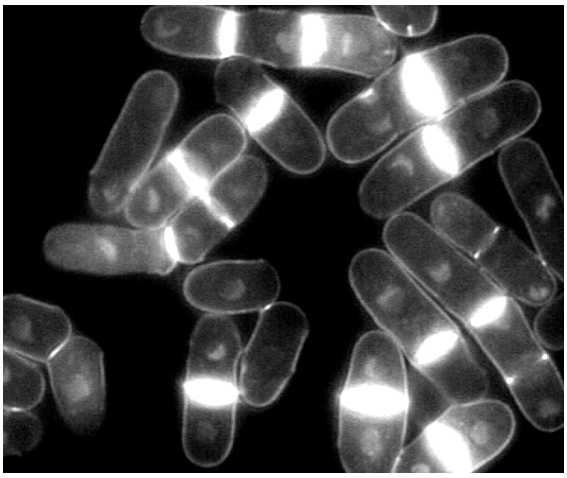
B



1. *exo70Δ* 2. *rho3Δ* 3. *exo70Δ rho3Δ*

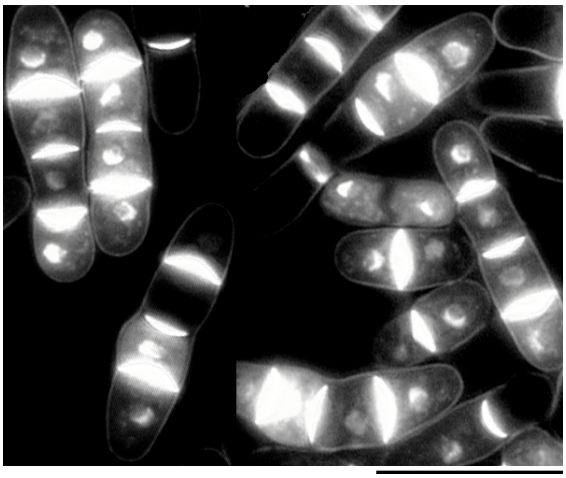
C

***exo70Δ rho3Δ*, 24°C**



DNA/Septum

***exo70Δ rho3Δ*, 32°C**



DNA/Septum

Figure 4.2.6 Two mutant forms of Rho3p. A dominant-active ((Rho3G25V) form and a dominant-inactive form of Rho3p (Rho3T30N) were constructed with mutations that are analogous to ras oncogene. The positions of the mutations were marked in the Rho3p amino acid sequence with a red and blue arrow head, respectively.

Fig 4.2.6

S. p. Rho3p

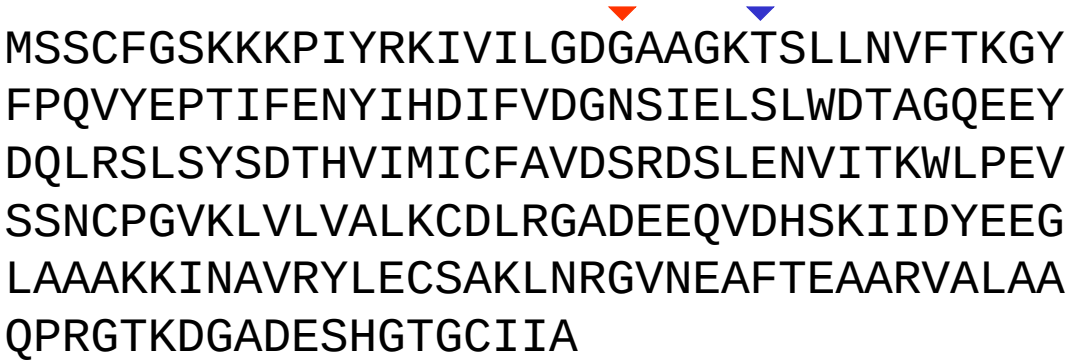
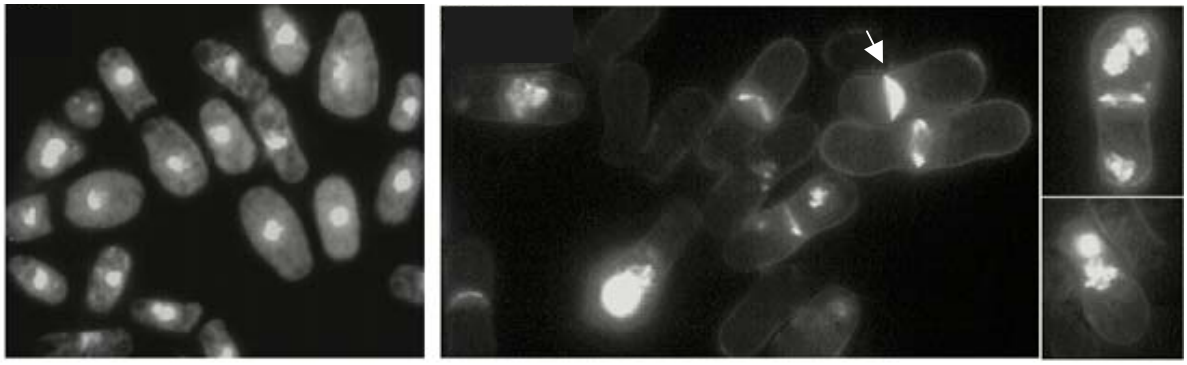


Figure 4.2.6.1 The phenotype of a dominant-active form of Rho3p. Cells expressing either Rho3-Val-25 under control of an nmt1 promoter were grown in the absence of thiamin at 30°C for 24 hrs, fixed and stained with DAPI and Calcofluor to visualize DNA and septum, respectively.

Figure 4.2.6.2 The phenotype of a dominant-inactive form of Rho3p. Cells expressing either Rho3-Asn-30 under control of an nmt1 promoter were grown in the absence of thiamin at 30°C for 24 hrs, fixed and stained with DAPI and Calcofluor to visualize DNA and septum, respectively.

Fig 4.2.6.1

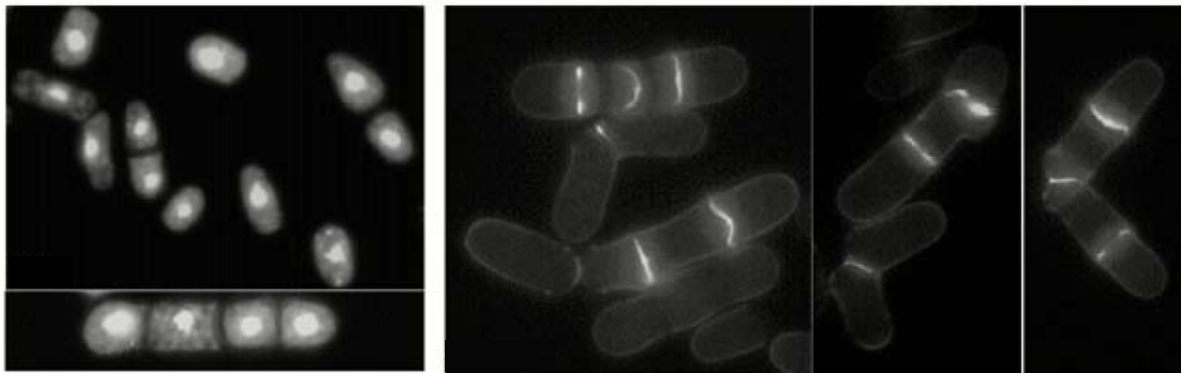
Rho3 G25V



DNA **Septa**

Fig 4.2.6.2

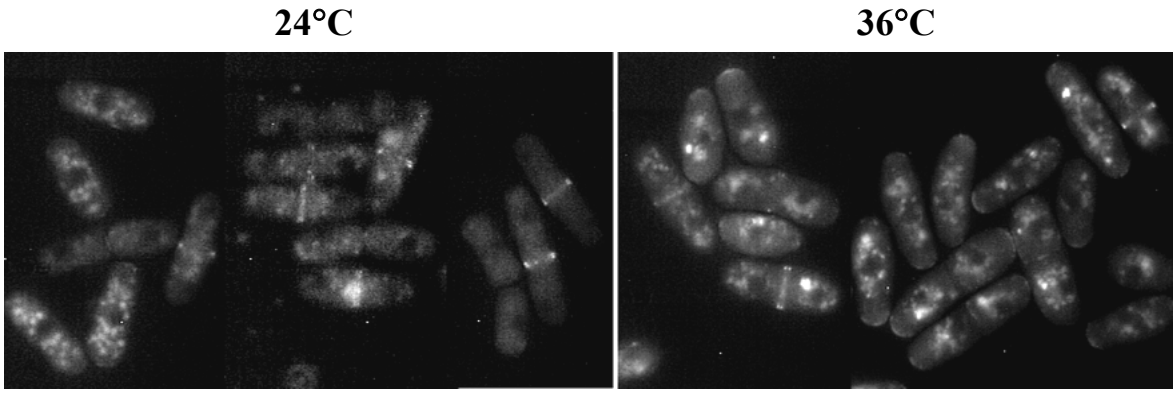
Rho3 T30N



DNA **Septa**

Figure 4.2.7 Sec8-GFP localization is *rho3* independent. *rho3*Δ cells expressing Sec8-GFP were grown at either 24°C or shifted to 36°C for 4 hrs, and examined for GFP epifluorescence. The Sec8p-GFP spot-like structures were also seen at 36°C in wild-type cells (data not shown).

Fig 4.2.7



Sec8-GFP, *rho3Δ* Sec8-GFP, *rho3Δ*

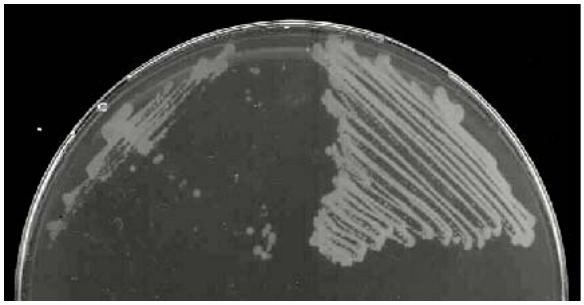
Figure 4.2.8.2 GFP-Rho3p expressed under nmt1 promoter localizes to the division site. (A) GFP-Rho3p expressed under nmt1 promoter is able to rescue *rho3Δ*. *rho3Δ* cells were transformed with plasmids expressing GFP alone (left-side colony) or GFP-*rho3* (right-side colony) from *nmt1* promoter at the repressed condition. Cells were grown at 24°C (not shown) and 36°C. (B) GFP-Rho3p localizes to the plasma membrane. The plasmid containing a fusion of with *GFP rho3* at its amino terminus and under control of the *nmt1* promoter was introduced into *S. pombe* wild-type cells. Cells were grown in the presence of thiamin at 30°C for overnight and visualized microscopically to examine the GFP epifluorescence signal.

Figure 4.2.8.3 GFP-Rho3p localization is partially dependent on functional Sec8p. Wild-type (upper panel) or *sec8*-1 (lower panel) expressing GFP-Rho3p were examined for GFP-Rho3p epifluorescence at 36°C.

Fig 4.2.8.2

A

rho3Δ*+GFP** **rho3Δ+GFP-*rho3



36°C

B

pREP1-GFP-*rho3*, 24 °C

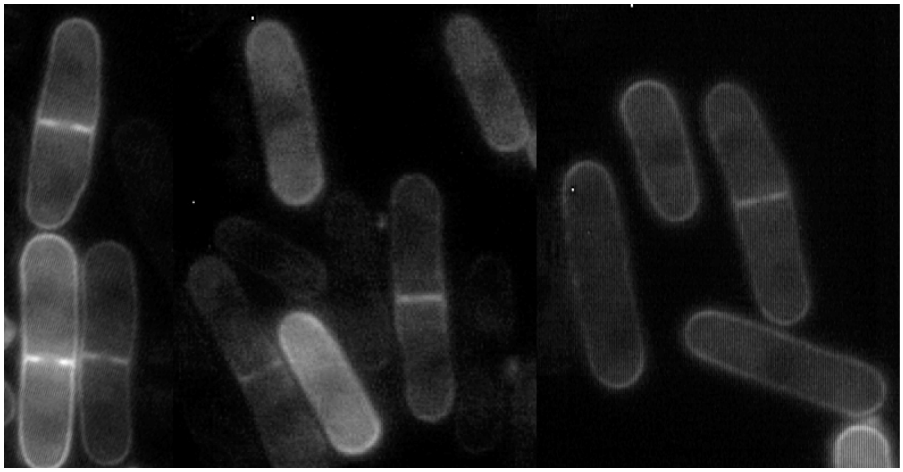
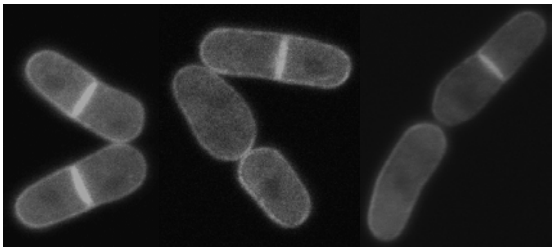


Fig 4.2.8.3

GFP-Rho3, 36°C ***sec8*-1, GFP-Rho3, 36°C**



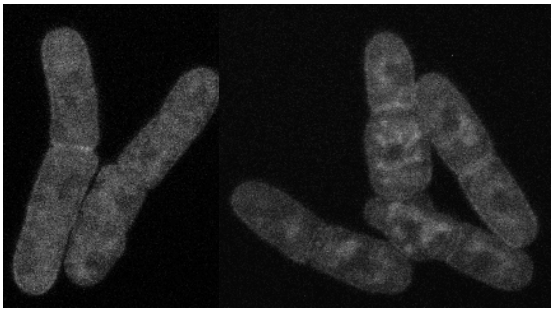


Figure 4.2.9.1 Rho3p is required for a late stage of secretory pathway. Electron microscopic analysis of *rho3Δ* cells. *rho3Δ* cells were grown at either 24°C (A) or shifted to 36°C for 4 hrs (B), fixed in permanganate and processed for electron microscopy analysis, respectively. Presumptive secretory vesicles are in dark staining and sized around 100 nm. Bar: 500 nm.

Fig 4.2.9.1

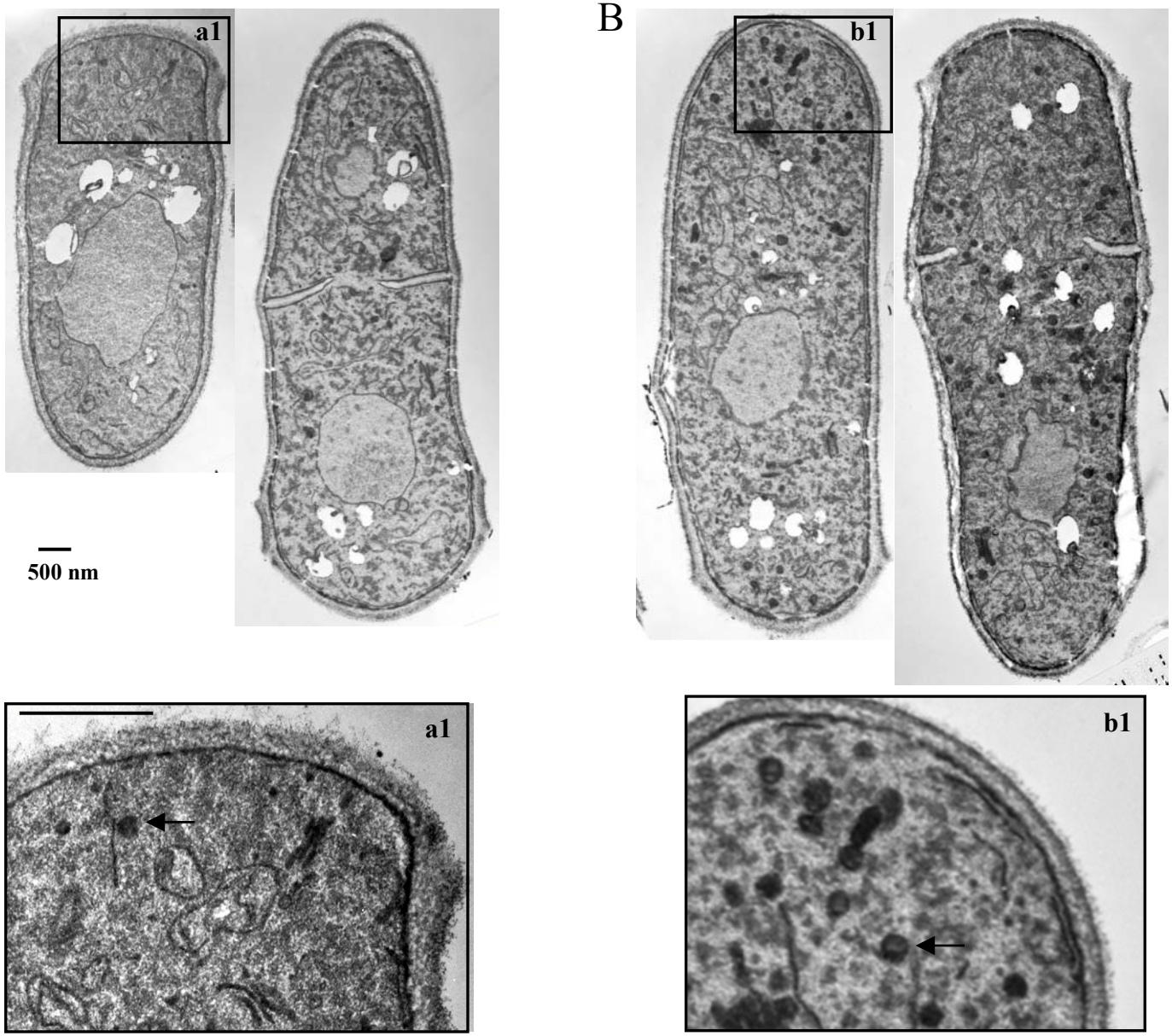
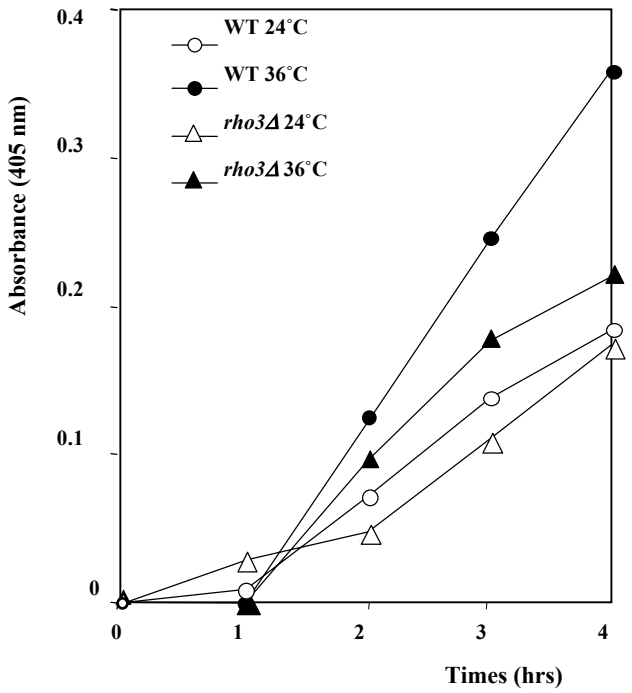


Figure 4.2.9.2 *rho3Δ* cells secrets lowered activity levels of acid phosphatase. Wild-type (circles) and *rho3Δ* (triangles) cells were assayed for secreted acid phosphatase activity at 24°C (open) and 36°C (filled)**.**

Fig 4.2.9.2


WT 24˚C
WT 36˚C
rho3Δ 24˚C
rho3Δ 36˚C
Absorbance (405 nm)
0.4
0.3
0.2
0.1
0
0
1
2
3
4
Times (hrs)

4.3 Discussion

4.3.1 Rho3p is a protein of Ras superfamily of small GTPase

The Ras GTPase superfamily of proteins are regulators of diverse biological processes including cell polarization, morphogenesis, cell growth, and development. By alternating between two different forms, an active GTP-bound state and inactive GDP-bound state, they act as molecular switches in response to different signals (Bourne et al., 1991). In yeasts, the Rho subfamily consists of six members, Cdc42p, Rho1p to Rho5p. In *S. cerevisiae*, Cdc42p, Rho3p and Rho4p play roles in polarized bud formation, while Rho1p, Rho2p and Rho5p are involved in synthesis and maintenance of the cell wall (Adamo et al., 1999; Adams et al., 1990; Cabib et al., 1998; Johnson and Pringle, 1990; Madaule et al., 1987; Matsui and Toh, 1992; Schmitz et al., 2002). In *S. pombe*, Cdc42p, Rho1p and Rho2p were shown to be important for polarized growth and morphogenesis (Fawell et al., 1992; Hirata et al., 1998; Miller and Johnson, 1994; Nakano et al., 1997; Nakano and Mabuchi, 1995). Recently, Rho3p, a novel Rho protein from *S. pombe*, was shown to interact with diaphanous /formin For3p to regulate polarized cell growth (Nakano et al., 2002) This study identifies the role for Rho3p in cell separation and exocytosis in *S. pombe*.

Our previous work established that the multiprotein exocyst complex is essential for cell separation in *S. pombe*. It was proposed that the exocyst mediates targeting of vesicles possibly containing primary septum degrading enzymes to the division site. This study shows that multicopy *rho3* is able to fully rescue the growth and cell separation defects of *sec8*-1 mutant at 36°C. However, overexpression Rho3p was unable to rescue a *sec8Δ* mutant, suggesting that high levels of Rho3p stimulates the residual function of the mutant Sec8-1p, rather than bypassing a requirement for

Sec8p. The synthetic-lethality between *sec8*-1 and *rho3Δ* mutant might also be explained by an additive lowering of exocyst function in the double mutant, although other possibilities cannot be ruled out. In this context, it is interesting to note that budding yeast Rho3p has also been shown to rescue conditional mutations in *sec8* and *exo70*. *rho3* was able to suppress a null mutant lacking Exo70p, an exocyst component only essential at higher temperatures, indicating that Rho3p might activate exocyst function through Sec8p, thus bypassing the requirement for Exo70p. However, it remains possible that Rho3p functions in a parallel pathway that is different from the exocyst pathway.

In budding yeast Rho3p has been shown to physically interact with the exocyst complex (Robinson et al., 1999). However, no physical interaction was detected between exocyst proteins (Sec8p, Sec10p and Exo70p) and Rho3p or Rho3G25V mutant in immunoprecipitation experiments (data not shown). Neither did any interaction of Sec8p with Rho3p was detected in a yeast two-hybrid system (data not shown). It is possible that this interaction might be transient and therefore difficult to observe.

Unlike in *S. cerevisiae*, Exo70p is non-essential in fission yeast. However, the *exo70*-null mutant leads to a temperature-sensitive growth phenotype. A double mutant *exo70Δ rho3Δ* had more severe cell separation phenotype than each single mutant, indicating that Rho3p might share a redundant function with Exo70p. The molecular basis of this interaction is currently unclear. It is possible that Rho3p or one of its effectors might play a role redundant with Exo70p, leading to the observed genetic interaction. Given similarities in the interactions between Rho3p and the exocyst

complex in budding and fission yeasts, it is possible that the exocyst function might be regulated similarly in other cell types as well.

4.3.2 Rho3p is a regulator of cell separation and exocytosis in *S. pombe*

Cells depleted of Rho3 protein show defects in cell separation, similar to that of exocyst mutants, suggesting that Rho3p is involved in this process. Consistently, overproduction of a dominant-inactive form of Rho3p (Rho3T30N) almost phenocopies *rho3Δ*. The localization of Rho3p to the division site, like exocyst proteins, is consistent with a role for this protein in cell division / separation. Currently it's not known whether Rho3p localization is dependent on F-actin. It will be interesting to know if Rho3p associates with secretory vesicles or if it regulates vesicle function. As with the exocyst null mutants, cell growth, elongation and cell polarity defects in *rho3* null mutant could not be observed, suggesting that Rho3p is important for cell separation but may not play a predominant role in cell elongation and division septum assembly. *rho3* is essential only at higher temperatures, whereas the exocyst complex is essential for cell viability at all temperatures. It is possible that Rho3p acts redundantly with other proteins, that might themselves be critical for viability at lower temperatures. Rho4p is an attractive candidate, but *rho3Δ rho4Δ* double mutant is viable (Wang et al, unpublished data), suggesting that additional Rho proteins such as Cdc42p (Zhang et al., 2001) and Rho1p (Guo et al., 2001) might also contribute to exocyst function, as shown recently in budding yeast.

I also showed that putative secretory vesicles accumulate in *rho3Δ* at the restrictive temperature, indicating that Rho3p is required for the targeting and/or fusion of secretory vesicles to the plasma membrane. A role for Rho3p in exocytosis was also

inferred from lowered acid phosphatase secretion in a *rho3*-deletion mutant compared to wild-type cells. Our data lead us to suggest that Rho3p may play a role in cell separation probably by targeting vesicles containing cell wall degrading enzymes to the division site via interaction with the exocyst complex. Interestingly, these vesicles are observed in both interphase and mitotic cells, suggesting that in addition to function during cell separation, Rho3p might also play a non-essential role in polarized cell growth / elongation. Consistent with this possibility is our finding that Rho3p is detected at the plasma membrane of interphase cells as well.

The polarized localization of Sec8p, Sec6p, Sec10p was not affected in *rho3Δ*, suggesting that exocyst proteins localized to the division site in a Rho3p independent manner. Thus, Rho3p modulates the exocyst function by mechanisms independent of those involved in their proper localization. It is conceivable that maximal activation / function of exocyst is achieved via interaction with Rho3p and other proteins redundant with it. Interestingly, the localization of Rho3p seems to be partially affected in *sec8*-1, indicating that the exocyst is required for the Rho3p localization. It will be important to further understand the mechanism of Rho3p localization in order to further speculate on its function in exocytosis. Future studies should unravel the molecular nature of interactions between Rho3p and the exocyst complex.

CHAPTER V GENERAL DISCUSSION

In the recent years, our understanding of the mechanisms regulating cytokinesis in *S. pombe* has advanced extensively by the isolation and characterization of cytokinesis defective mutants and the analysis of the corresponding wild-type gene products. Most of the studies have been focused on understanding the process of assembly of the actomyosin ring and the division septum. However, the mechanism of new membrane addition during cytokinesis as well as cell separation is only poorly understood in this yeast.

In animal cells, new membrane addition during cytokinesis requires fusion factors, e.g. syntaxins, to mediate membrane fusion at the division site, while during cytokinesis of plant cells and budding yeast cells, both new membrane addition and the delivery of cell wall synthesizing enzymes to the plasma membrane of the division site need exocytic factors, e.g. syntaxins in plant and budding yeast cells and the exocyst in budding yeast (Bowerman and Severson, 1999; Smith, 1999). Presently, it is unclear if the exocyst proteins play a role in cytokinesis of plant cells (Elias et al., 2003). As one would expect, fission yeast also requires targeted membrane addition as well as cell wall assembly during cytokinesis, which are likely to be dependent on exocytic factors. The study of Cps1p, an integral membrane localized 1,3-β-glucan synthase required for septum formation, provides some clues to the role of targeted exocytosis in completion of cytokinesis (Liu et al., 2002; Liu et al., 1999). Treatment of *S. pombe* cells with BFA, a drug that blocks membrane trafficking from ER to Golgi apparatus, results in cells unable to form a septum and blocked with two nuclei and a stable actomyosin ring, phenocopying a phenotype of a *cps1*-191 mutant (Liu et al., 2000; Jianhua Liu, personal communication), further indicating a requirement of the

secretory pathway for septum formation. Although cytoskeletal factors and regulatory proteins involved in cytokinesis including actomyosin ring components (that are required for actomyosin ring assembly), Cdc15p (that is required for the redistribution of F-actin patches to the division site) and SIN components (that regulate septum formation) have been identified, there are no report on proteins that directly mediate the targeted delivery of membranes and cell wall biosynthetic proteins to the division site during cytokinesis through exocytosis. Exocytic factors that are important for these exocytic events in other organisms, such as syntaxins, rab proteins and the exocyst, were not identified in previous genetic screens for cytokinesis mutants. It is possible that given the multi-functional nature of these proteins, appropriate temperature-sensitive alleles with desired characters are rarely observed.

This study attempted to investigate whether the exocyst, a multisubunit protein complex, that participates in exocytosis during cell growth and cytokinesis, in budding yeast (Guo et al., 1999), is involved in cytokinesis in fission yeast. Interestingly, work presented in this thesis have demonstrated that a requirement for the exocyst for cell separation, but not for the delivery of new membranes during cytokinesis, or for the formation of the division septum in fission yeast. Given that the exocyst mutants accumulate ~100 nm vesicles in the cytoplasm, it is likely that the fission yeast exocyst complex, similar to the budding yeast exocyst complex, functions in a late step of the secretory pathway. It is likely that the *S. pombe* exocyst may specifically target a class of vesicles that contain cell wall degrading enzymes to the plasma membrane of the division site. A likely cargo of such vesicles is Eng1p, a 1,3-β-glucanase involved in cell separation (Martin-Cuadrado et al., 2003). Current studies by Martin-Cuadrado

(personal communication) are aimed at answering if extracellular secretion and localization of Eng1p is affected in fission yeast exocyst mutants.

The results from this study are somewhat different from those reported from studies in the budding yeast. While *S. cerevisiae* exocyst is essential for all exocytic events during polarized cell growth, membrane assembly during cytokinesis and septum formation (TerBush et al., 1996), in fission yeast only cell separation is severely affected in exocyst mutants, and polarized cell growth, membrane assembly during cytokinesis and septum formation continue in the absence of or with minimal and experimentally undetectable amounts of exocyst function. It remains possible that all the mutants analyzed in this study are not completely depleted of exocyst function leading to the observed phenotypes. If this were the case, it can be concluded that cell separation is most sensitive to the requirement for optimal exocyst function. Construction and analysis of a series of rapidly inactivating loss-of-function mutants in the exocyst should allow us to address whether the *S. pombe* exocyst has any role in polarized cell growth.

The fission yeast exocyst localizes to the division site during cytokinesis but this localization is not perturbed upon the treatment of cells with BFA, indicating that it is independent of the BFA-dependent secretion. Given the similarities between septation process in the two yeasts, and the finding that secretion plays a critical role in new membrane addition and septum formation during cytokinesis in *S. cerevisiae*, it is possible that in *S. pombe*, there are two different exocytic pathways mediating the trafficking of secretory vesicles during cytokinesis. While the exocyst-independent secretion pathway may be crucial for septum assembly and new membrane formation,

secretion mediated by the exocyst may be essential only for cell separation. Recently, it was reported that filipin, a fluorescent drug that binds to sterol, marks the membrane domains during cytokinesis in *S. pombe* and its localization to the division site is abolished upon treatment with BFA (Wachtler et al., 2003). Genetic screens searching for mutants with a phenotype similar to that of BFA-treated cells (BFA phenotype) using filipin as a marker for membrane domain during cytokinesis, should identify molecular components of the exocyst independent pathway that is important for aspects of cytokinesis such as membrane and division septum assembly. Another possibility is that in *S. pombe* membrane vesicles that are required for septum formation are not transported directly from the Golgi apparatus to the plasma membrane, but may adopt a detour from the Golgi apparatus to another compartment i.e. endosome, on their way to the plasma membrane.

I have also analyzed *S. pombe* Rho3p, a member of ras GTPase family, which affects cell separation by modulating the exocyst function. Rho3p interacts genetically with Sec8p and has a role in cell separation, similar to the exocyst. The localization of the exocyst proteins at the division site is normal in *rho3Δ*, suggesting that Rho3p regulates the exocyst in a manner distinct from involvement of localizing the exocyst. Overproduction of Rho3p rescues the cell separation defects associated with *sec8*-1, indicating that Rho3p may stabilize the exocyst complex. The accumulation of putative secretory vesicles in the cytoplasm of *rho3*Δ cells suggests a role for Rho3p in exocytosis, which also further suggests a requirement for exocytosis during cell separation.

In fission yeast, several other proteins important for cell separation have been identified. For example, septins from fission yeast are also required for cell separation (Berlin et al., 2003; Tasto et al., 2003). Interestingly, the mammalian septins interact with Sec6/8 complex to effect vesicle trafficking to the plasma membrane of neurons (Hsu et al., 1998). However, the exocyst does not seem to interact with septins in *S. pombe* (Chapter 3.2.13), suggesting that they function independently of each other in cell separation. Unlike the exocyst which is essential for cell separation, *rho3* is a non-essential gene and *rho3*Δ cells only have a mild defect in cell separation, suggesting that additional protein(s) may play a role redundant with Rho3p in cell separation. Rho4p, another non-essential rho GTPase, has recently been found to be involved in cell separation, similar to Rho3p (Nakano et al., 2003; Santos et al., 2003). It is possible that Rho4p might have a redundant function with Rho3p in cell separation. The genetic interaction between *rho3* and *rho4* remains to be tested. Currently it is unknown whether Rho4p also regulates the exocyst. Future studies should explore the relationships between Mid2p, septins, Rho3p, Rho4p, the exocyst and Eng1p and how they collaborate and contribute towards effecting cell separation.

Concluding remarks and future directions

In this study, the essential role of a multi-protein complex, the exocyst, in cell separation is described. Exocyst proteins localize to putative regions of active exocytosis and form a complex *in vivo* in *S. pombe*. Exocyst mutants appear to be able to elongate and assemble division septa, but are defective in cell separation. I therefore propose that the fission yeast exocyst may be involved in targeting of enzymes responsible for septum cleavage. The interaction of the exocyst with Rho3p,

a member of the Rho family of small GTPase, suggests that *S. pombe* Rho3p may regulate cell separation by modulating exocyst function.

Although the knowledge of cytokinesis in *S. pombe* has been expanded dramatically in the last decade, intriguing and fundamental questions related to membrane trafficking during *S. pombe* cytokinesis remain to be answered. For example, What are the dynamics of membrane insertion during cytokinesis? Does cytokinesis require both new membrane insertions at the division site as well as targeting of proteins and lipids from other domain of the plasma membrane? Are they mediated by different mechanisms? Are there different categories of secretory vesicles transported via different routes during cytokinesis? Are there proteins involved in exocyst – independent secretion that may effect new membrane or septum formation during cytokinesis? Are other membrane trafficking events, such as endocytosis and vesicle mediated recycling, involved in cytokinesis? How is new membrane formation during cytokinesis coordinated with the constriction of the actomyosin ring and the formation of division septum? Is the new membrane addition during cytokinesis being monitored and are there any sensor proteins to detect the failure of secretion during cytokinesis? Further studies that attempt to address these questions may lead to a better understanding of the mechanism of cytokinesis. Given the conservation of several aspects of the process of cytokinesis between fission yeast and other organisms, insights obtained from studies in fission yeast should be applicable to other organisms as well and may help understand the general process of cytokinesis.

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